The role of microglia phenotypes in modulating CD4$^+$ T cell responses

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Zusammenfassung

Die Invasion von Leukozyten in das zentrale Nervensystem (ZNS) ist ein wesentlicher Bestandteil bei der Pathogenese von Hirnverletzungen sowie akuten und chronischen Entzündungsvorgängen im Gehirn. Bisher ist nur wenig über gegenregulatorische Mechanismen der lokalen, angeborenen Immunzellen bekannt, die bei Entzündungen im ZNS eine Rolle spielen können. Mikrogliazellen, die überwiegende Population immunkompetenter Zellen des ZNS, stellen die erste Verteidigungslinie im Hinblick auf Verletzungen und Erkrankungen des Gehirns dar. Im Rahmen vieler neurodegenerativer Erkrankungen wird die Zerstörung von Neuronen, aber auch die kollaterale Gewebsschädigung auf die Aktivierung der Mikrogliazellen zurückgeführt.

Die vorliegende Arbeit beschreibt erstmalig einen regulatorischen Aktivierungszustand der Mikroglia ($CD40^{dim}CD86^{dim}IL\text{-}10^{high}$), der zur Induktion regulatorischer $Foxp3^+$ T-Zellen (Treg) führt. Die Stabilität und funktionelle Aktivität Mikroglia-induzierter regulatorischer T-Zellen konnte sowohl *in vitro* als auch *in vivo* gezeigt werden. *In vitro* inhibierten sie die Proliferation antigen-spezifischer Effektorzellen, *in vivo* führte ein adoptiver Transfer der regulatorischen T-Zellen zur Abmilderung des Krankheitsverlaufes experimentell induzierter, autoimmuner Enzephalomyelitis (EAE). Mikrogliazellen unterstützten sowohl die Proliferation bereits ausgebildeter regulatorischer T-Zellen als auch deren Differenzierung aus naiven T-Zellen. Die Induktion regulatorischer T-Zellen durch Mikroglia war Major Histocompatibility Complex (MHC)-II-abhängig und antigenspezifisch. Für Untersuchungen zur *in vivo* Relevanz wurden MHC-II-chimäre Mäuse generiert und eine Läsion im entorhinalen Kortex gesetzt. Fehlte MHC-II in ZNS-residenten Zellen, wurden weniger regulatorische T-Zellen pro Leukozyt in die lädierten Hemispheren rekrutiert.

Zusammenfassend demonstrieren diese Ergebnisse das Modulationspotential von Mikrogliazellen auf die $CD4^+$ T-Zellantwort. Die Mikroglia-induzierte Differenzierung und Proliferation von $Foxp3^+$ regulatorischen T-Zellen ist ein möglicher Mechanismus der Regulation von Entzündungsvorgängen im ZNS durch Mikrogliazellen.

Summary

The invasion of leukocytes into the central nervous system (CNS) is a key event in the pathogenesis of CNS injury and acute or chronic inflammatory neurological diseases. However, regulatory mechanisms of local innate immune responses that limit CNS inflammation are only poorly understood. Microglia are the predominant innate immune cells of the brain and present the first line of defence in CNS injury or disease. In the context of neurodegenerative disease, microglia activation accounts for collateral tissue damage and neurodestruction.

This thesis for the first time describes a regulatory microglia phenotype ($MHCII^{+}CD40^{dim}CD86^{dim}IL\text{-}10^{high}$) that induced a strong $Foxp3^{+}$ regulatory T cell (Treg) response. Microglia-induced Treg cells were stable and functionally active *in vitro* by inhibiting antigen-specific proliferation of effector T cells and *in vivo*, by attenuating experimental autoimmune encephalomyelitis (EAE) disease course after adoptive transfer. The data also suggested that regulatory microglia can mediate both, proliferation of $Foxp3^{+}$ Treg cells and *de novo* differentiation from naive $CD4^{+}$ T cells. Microglia-mediated Treg induction was proven to be MHCII and antigen-dependent. Using entorhinal cortex lesion (ECL) as a brain injury mouse model, diminished $Foxp3^{+}$ Treg cell recruitment per infiltrated leukocyte in chimeric mice lacking MHCII specifically in the CNS was demonstrated, indicating *in vivo* relevance of antigen presentation by brain resident cells.

Taken together, these findings demonstrate that microglial cells can directly modulate $CD4^{+}$ T cell responses by regulating molecule levels for efficient antigen presentation and levels of secreted cytokines and chemokines. Microglia-mediated differentiation and proliferation of $Foxp3^{+}$ Treg cells can be one of the mechanisms how microglia contribute to local immune homeostasis and limit CNS inflammation.

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Abbreviations

Aβ	Amyloid β peptide
AD	Alzheimer's disease
β-Me	2-Mercaptoethanol
BBB	Blood-Brain Barrier
BCSFB	Blood-Cerebrospinal Fluid Barrier
BM-DC	Bone Marrow derived Dendritic Cells
BM-M	Bone Marrow derived Macrophages
CD	Cluster of Differentiation
CFA	Complete Freund's Adjuvant
CLN	Cervical Lymph Nodes
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DMEM	Dulbecco's modified Eagle's Medium
DNA	Desoxyribonucleic Acid
EAE	Experimental Autoimmune Encephalomyelitis
ECL	Entorhinal Cortex Lesion
EDTA	Ethylene Diamine Tetraacetate
EGFP	Enhanced GFP
ELISA	Enzyme-linked Immunosorbent Assay
FACS	Fluorescence activated Cell Sorter
FCS	Fetal Calf Serum
Foxp3	Forkhead box P3
g	Gravitational acceleration
GFP	Green Fluorescent Protein
GMCSF	Granulocyte-Macrophage Colony-Stimulating Factor
HBSS	Hank's buffered Salt Solution
HPA	Hypothalamic Pituitary Adrenal
IFN	Interferon
IL	Interleukin
iTreg	Inducible regulatory T cell
LPS	Lipopolysaccharide
mAb	Monoclonal Antibody
MBP	Myelin Basic Protein
MCAO	Middle Cerebral Artery Occlusion

MCSF	Macrophage Colony-Stimulating Factor
MHC	Major Histocompatibility Complex
min	Minute
MOG	Myelin Oligodendrocyte Glycoprotein
mRNA	Messenger RNA
MS	Multiple Sclerosis
NO	Nitric Oxide
nTreg	Natural occurring regulatory T cell
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
SEM	Standard Error of the Mean
TCR	T Cell Receptor
TGF-β	Transforming Growth Factor β
TLR	Toll-Like Receptors
TNF	Tumor Necrosis Factor
Tris	Trisaminomethane
U	Units
w/o	Without
WT	Wild-Type

1 Introduction

Experiments showing foreign graft acceptance had once formed the idea of the central nervous system (CNS) as an organ ignored by the immune system. Being isolated from the immune system by the blood-brain barrier (BBB) and by the blood–cerebrospinal fluid barrier (BCSFB) and lacking conventional lymphatics, the CNS compartment was considered as an immune privileged site. Minimal major histocompatibility complex (MHC) class II expression on parenchymal cells, low cytokine and chemokine levels and low T cell numbers in healthy brains supported this idea[1]. However, increasing evidence suggests a new concept of the interplay between the immune and the central nervous system and eventually led to revision of this long-standing concept of the CNS immune privilege.

The BBB is a complex structure consisting of specialized capillary endothelial cells, basal laminas and adjacent pericytes, astrocytes, and microglia forming the functional neurovascular unit. CNS endothelial cells differ from non-CNS endothelial cells by forming tight junctions and a deficiency in transcellular transport systems, e.g. pinocytotic vesicles. As a result, the BBB is a physical, metabolic and immunological barrier limiting the access of circulating blood cells and soluble factors, except of those that are small and lipophillic[2]. Pathological conditions that affect BBB integrity and allow immune cell infiltration are Alzheimer's and Parkinson's disease (BBB disruption), inflammation (increased BBB permeability), multiple sclerosis (disruption of tight junctions), stroke (BBB disruption) or brain trauma (BBB breakdown)[3]. Contrary to historical thinking, latest studies show, that even under non-pathological conditions, peripheral immune cells are able to transmigrate across the intact BBB[4–6]. CNS immune surveillance is now considered a normal ongoing physiological process[7].

In the healthy state, the local CNS milieu is characterized by very low levels of immunologically relevant molecules and, in marked contrast to the historical view of immunologic incompetence, this suppressed CNS microenvironment is actively maintained by functional neurons. In turn, neurodegenerative pathology is often accompanied by inflammatory activity, such as activation of brain resident immune cells, up-regulation of cytokines and chemokines and the recruitment of leucocytes into the brain[8]. Compared to peripheral tissues, the CNS is less efficiently drained by lymphatic vasculature. However, T cells specific for brain antigens are found in the normal lymphatic T cell repertoire[9,10] and several outflow pathways connecting the CNS with lymphatics have been described[11] allowing the transport of e.g. brain antigens to CNS draining lymph nodes.

Therefore, the concept of the CNS as immunologically inert and immunologically separated from the peripheral immune system has to be rejected and replaced by the notion of a dynamically interacting and complex relationship between the CNS and the immune system.

1.1 Blood-brain and blood-cerebrospinal fluid barriers

Although immune cell trafficking into the brain is strictly controlled by the brain barriers, there is rising evidence for continuous leukocyte trafficking into the healthy CNS for routine immunosurveillance[12–14]. The blood-brain barrier (BBB) is anatomically organized around the brain parenchyma. The choroid plexus, where the cerebrospinal fluid (CSF) is synthesized and meningeal venules are surrounded by the blood-CSF barrier (Fig. 1.1)[15]. The subarachnoid space, between the arachnoid mater and the pial membrane, is filled with circulating CSF. Depending on their routes of entry, T cells can either enter the subarachnoid space by migrating from the choroid plexus stroma across the blood-CSF barrier, or they cross the BBB and enter the perivascular space that surrounds the postcapillary venules (Fig. 1.1). Tight-junctions regulate the permeability properties of both, the blood-brain barrier formed by endothelial cells, as well as the blood-CSF barrier, formed by epithelial cells.

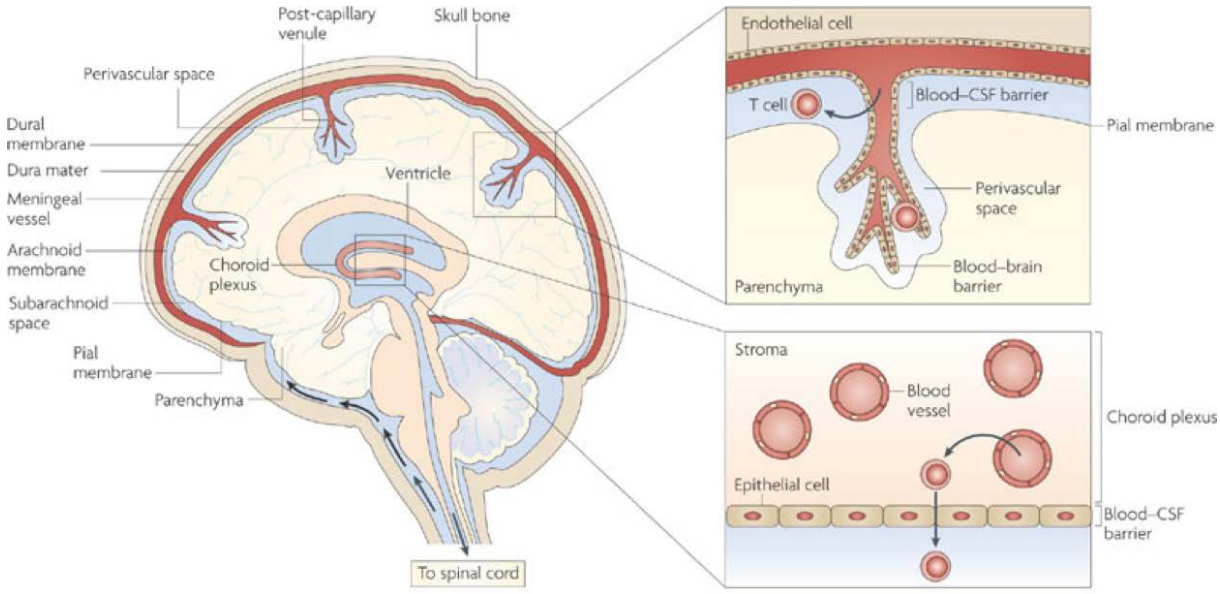

Fig. 1.1 Anatomical organization of the brain barriers.
The brain parenchyma is surrounded by the blood-brain barrier (BBB). The blood-CSF barrier (BCSFB) surrounds choroid plexus and meningeal venules. CSF circulates in the subarachnoid space, between arachnoid mater and pial membrane. Possibe routes of T cell entry are across the BCSFB into the subarachnoid space or across the BBB into the perivascular space. (Figure adapted from *J. Goverman, Nature Reviews Immunology 9, 393-407, 2009*)[15].

1.2 Cells and microenvironment of the brain

The CNS is composed of two kinds of specialized cells: neurons and glial cells. Glial cells, known as supporting cells, vastly outnumber neurons in the brain, indicating how much support a neuron needs to process and transmit information through the nervous system. Besides ensuring structural integrity, glial cells provide metabolic functions, assist in repair and maintenance of the nervous system, clear waste or are involved in key developmental processes, to name but a few of their functions. Astrocytes, oligodendrocytes, and microglia are the three types of glial cells that support the health of neurons in the CNS, the latter being the focus of this thesis.

Oligodendrocytes and astrocytes constitute macroglia[16]. Astrocytes, which are now thought to be involved in almost all aspects of brain function[17], were discovered to even release gliotransmitters, thereby facilitating neuronal communication. The principle function of oligodendrocytes is to form the myelin sheath maintaining long-term axonal integrity[18]. Microglia, the resident innate immune cells of the brain, are the smallest of the supporting glial cells and found throughout the CNS.

The healthy CNS is often termed immunosuppressive, referring to the hostile environment created for infiltrating cells of the adaptive immune system[19]. Besides low expression of MHC class II and pro-inflammatory cytokines, the neuronal microenvironment is tightly controlled and modulated by immunosuppressive cytokines (TGF-β, IL-1) and neurotrophins. Immunosuppressive factors produced by neurons such as neurotrophin-3 (NT-3), nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are thought to limit the activation of astrocytes and microglia[20].

1.3 Microglia – Immune sentinels of the CNS

Microglia are the predominant innate immune cells in the brain and therefore present the first line of immune defense whenever there is injury or disease[21,22]. Depending on the specific region of the CNS, microglia constitute about 5-20% of total glial cells and vary in density and morphology[23,24]. Compact microglial cells with short, thick processes are found in circumventricular organs, longitudinally branched cells in the fibre tracts and radially branched cells in all areas of the grey matter[23]. The tissue of origin and the specific lineage of microglia and their progenitors was a subject of debate for decades. In contrast to neuroectoderm-derived neurons, astrocytes and oligodendrocytes, microglial progenitors are now thought to arise from peripheral mesodermal (myeloid) tissue[25]. However, whether circulating monocytes and/or myeloid progenitor cells contribute to the repopulation of steady-state parenchymal microglia in the adult CNS is still an unsolved question[24].

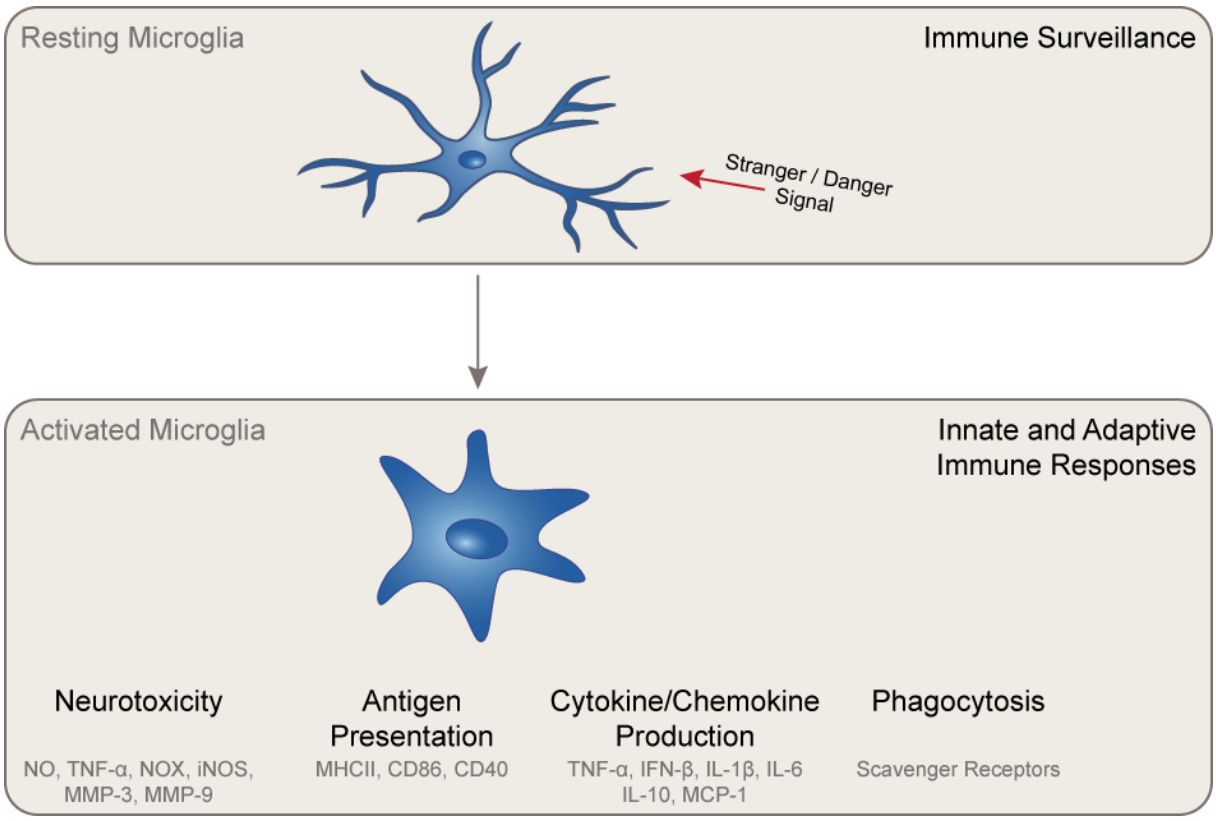

Fig. 1.2 Microglia activation.
Upon stranger (invading pathogens) or danger (trauma, neurodegeneration, ischemia, inflammation or altered neuroactivity) signals microglia undergo a characteristic morphological change. Resting microglia, characterized by ramified morphology with fine processes, participate in immune surveillance and display an immunmodulatory down-regulated phenotype. Activated microglia aquire amoeboid morphology and participate in innate and adaptive immune responses (by phagocytosis, cytokine/chemokine production and antigen presentation) or mediate neurotoxicity by release of neurotoxic factors.

In the healthy brain, microglia are continually palpating and surveying their local environment[26], exhibiting a typical ramified morphology with small somata and fine processes with secondary and tertiary branches (**Fig. 1.2**). In this appearance, microglia exhibit an actively repressed or down-regulated phenotype, clearly distinct from other macrophage populations, which is usually termed "resting" state. In response to invading pathogens, inflammation, trauma, ischemia, neurodegeneration or altered neuronal activity, microglia rapidly undergo changes resulting in diverse functional phenotypes and amoeboid morphology (reactive microgliosis). Depending on the stimulus confronted with, activated microglia accumulate at sites of tissue damage, up-regulate various cell surface antigens, produce cytokines and chemokines or increase phagocytosis, as described in more detail in the following section. This rapid shift from a resting to an activated state, even in response to poor stimuli, is a hallmark of microglial cells, which are therefore termed "immune sentinels" of the CNS.

Upon activation, microglia participate in both innate and adaptive immune responses[24] similar to peripheral macrophages. Activated microglial cells share several phenotypical and functional characteristics of circulating and tissue-resident macrophages, leading to the common designation: resident macrophages of the brain. Indeed, there is currently no cell surface marker available that reliably distinguishes brain resident microglia from peripheral macrophages. Particularly under pathological conditions associated with leukocyte entry into the CNS, a clear discrimination between brain resident microglia and brain infiltrating phagocytic cells remains difficult. The integrins CD11a and CD11b, LPS co-receptor CD14, leukocyte common antigen (CD45) and F4/80 (EMR1) are common cell surface markers of both, murine microglia and macrophages. For flow cytometry, the combination of CD45 and CD11b is often used to roughly distinguish between microglia ($CD11b^{+}CD45^{dim}$) and macrophages ($CD11b^{+}CD45^{high}$)[27]. In turn, many antigens typically found on other macrophage populations, such as MHC class II, ICAM-1 or CD40, are missing or expressed at low levels on microglia of the healthy CNS[1].

1.3.1 Microglia in health and disease

The term “microglial activation” implies that resting microglia are without functional importance. However, microglia of the healthy CNS constantly monitor their environment and adjacent cells using their fine, motile processes[28]. The term “resting” state refers to their immunomodulatory down-regulated phenotype. Distinct from other macrophage populations, resting microglia exhibit only low levels of MHC II and co-stimulatory molecules and secrete themselves immunosuppressive factors (TGF-β, BDNF, NGF)[29]. Under steady-state conditions their down-regulated phenotype is actively maintained by functional neurons, e.g. cell-cell contact (CD47-CD172, CD200-CD200R, CD22-CD45)[30] and secreted factors (CX3CL1)[31]. In addition, astrocytes produce immunosuppressive IL-4, IL-10 and TGF-β[32,33]. Performing continuous surveillance of their environment, microglia are key players in maintaining CNS homeostasis. Any disturbance in the CNS, changes in structural integrity, invasion of pathogens or alterations in microenvironment, can evoke a microglial response.

“Microglial activation” is a term widely used in the context of CNS inflammation or neurodegenerative disease – most often without further definition. However, recent studies describe a phenotypic and functional plasticity of microglia that is dynamic and finely graded. The concept of microglia activation therefore needs to be revised, assigning a clear definition to the respective phenotypes. In the following, different microglia activation phenotypes will be introduced in the context of neurodegenerative disease or CNS injury.

Infections of the CNS result in innate and adaptive immune responses initiated by microglial cells. Microglia sense invading pathogens (e.g. bacteria, viruses and protozoa) and related particles by Toll-like receptors (TLRs), a major family of pattern recognition receptors. The

entire complement of TLR 1-9 are expressed by microglia[34]. Following pathogen recognition, microglia release pro-inflammatory mediators, such as TNF-α, IFN-β, IL-1β or IL-6[34], and chemokines (CXCL-10, MCP-1) to recruit cells of the adaptive immune system (e.g. T lymphocytes and monocytes) to the site of infection for pathogen clearance[35]. It is proposed that the microglia-mediated defense against CNS invading pathogens leads to collateral tissue damage and neurodegeneration through secretion of neurotoxic factors (NO, TNF-α) and leukocytes recruitment[36].

The role of microglia in *Alzheimer's Disease (AD),* a progressive*,* irreversible neurodegenerative disease*,* is discussed controversially. Activated microglia are closely associated with β-amyloid (Aβ) plaques, which are, together with neurofibrillary tangles, a histopathological hallmark in AD brains. Microglia can respond to amyloid-β deposition by up-regulating phagocytic activities (e.g. expression of scavenger receptors) resulting in Aβ clearance, but at the same time, they can locally secrete neurotoxic substances (inflammatory cytokines, reactive oxygen species, proteinases, and complement proteins) leading to tissue damage of surrounding areas[37,38]. In addition, several groups showed that activated microglia that co-localized with Aβ plaques also up-regulated MHCII[39,40]. Immunotherapy of Alzheimer's disease therefore focuses on activating microglia to decrease the amyloid-β load without inducing neurotoxic inflammation that occurs secondary to amyloid-β deposition[37].

Multiple Sclerosis (MS) is a heterogeneous autoimmune disease that is characterized by severe CNS inflammation, demyelination and axonal loss resulting in a variety of neurological symptoms[41,42]. Autoreactive, myelin-specific T cells of the CD4 T cell lineage, mainly Th1 and Th17 cells, are thought to mediate MS pathology by either promoting the accumulation of inflammatory immune cells (typical white matter lesions) or directly damaging oligodendrocytes and neuronal cells. It is generally thought that Th1 and also Th17 cells, directed against myelin antigens such as proteolipid protein (PLP), myelin basic protein (MBP), myelin associated protein (MAG) or myelin oligodendrocyte glycoprotein (MOG), enter the brain were they re-encounter CNS antigens presented by microglia and/or perivascular macrophages and initiate a destructive inflammatory cascade[43,44]. Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model of MS. EAE can be either induced actively by immunization with myelin antigens or peptides (MOG, PLP, MBP) in complete Freund's adjuvant, or passively by adoptive transfer of activated encephalitogenic T cells isolated from immunized animals[45]. EAE studies in MHC-mismatched bone marrow chimeric mice demonstrated that brain resident microglia up-regulate CD45, MHCII, CD86, CD40 and CD11c as a sign of activation and undergo proliferation very early in disease progression[46]. Activated microglial cells not only co-localize

with infiltrating leukocytes in inflammatory lesions[46], but are also found throughout the brain during disease progression[47,48]. Whether myelin-antigen presenting microglia migrate out of the brain and reach brain draining cervical or deep cervical lymph nodes is still controversially discussed.

Moreover, synaptic pathology was shown to correlate with the presence of activated microglia[47]. This is supported by a study showing that inactivation of microglia, using CD11b-HSVTK mice, inhibits EAE, suggesting that the microglia compartment provides a potential therapeutic target[49]. In sum, microglia activation towards antigen presentation is generally thought to contribute to MS pathology.

Within a few hours after an *ischemic stroke* analysis of blood, spleen and thymus show marked and long-lasting lymphopenia together with impaired cellular immune functions as part of a stroke-induced immunodeficiency syndrome (SIID)[50]. Possible explanations for SIID involve elevated lymphocyte apoptosis in spleen and thymus, a shift to an anti-inflammatory cytokine response in the peripheral organs or increased frequencies of regulatory T cells[50]. In addition, brain ischemia and resulting cell death induce hyperactivity of the sympathetic nervous system and the hypothalamic–pituitary–adrenal (HPA) axis, thereby increasing the risk of infectious complications[51]. The local immune responses to stroke include the release of endogenous molecules termed damage-associated molecular patterns (DAMPs) from dying cells, the activation of microglia and astrocytes and the recruitment of leukocytes[52]. Microglial cells rapidly migrate to the affected site, undergo proliferation and release neurotoxic mediators such as superoxide, nitric oxide (NO), proteases (metalloproteases MMP-3, MMP-9) and proinflammatory cytokines (TNFα, IL-1β, IL-6)[53]. Whether or how microglia directly contribute to the systemic immunodepression after stroke e.g. by secreting mediators that enter the systemic circulation, is still a matter of debate. Besides the fact that post-ischemic inflammation exacerbates ischemic injury there is emerging evidence that some aspects of immune responses might promote tissue repair and recovery[52].

CNS injury mostly results in axonal degeneration, cell death, destruction of the BBB, massive release of self-antigens and expansion of myelin-specific T cells in local lymph nodes[54]. In experimental lesion models such as entorhinal cortex lesion (ECL), facial nerve crush, spinal cord injury (SCI) or traumatic brain injury (TBI), microglia were shown to be activated within one hour after injury[55] and remain activated up to one or two weeks after injury[2,56]. CNS tissue damage induces microglia to migrate to the lesion site, increase in number[57], phagocytose neuronal debris and present antigens in a MHCII dependent way[58]. In addition to general leukocyte recruitment (e.g. neutrophils, monocytes), myelin-specific lymphocytes expand in draining lymph-nodes[59], subsequently invade regions of degeneration[58,60] and mediate secondary white matter damage. However, brain injury does not evoke destructive immunity, such as observed in MS or EAE. These findings indicate that inflammation in

regions of degeneration is tightly regulated and controlled, implying active maintenance of immune tolerance[54].

In sum, microglia contribute to both innate and adaptive immunity. Microglial cells can migrate to regions of tissue damage, undergo proliferation, increase phagocytic activity to clear debris, up-regulate molecules for effective antigen presentation, secrete cytokines and chemokines in order to regulate recruitment of leukocytes to the CNS or release reactive oxygen species and proteases (**Fig. 1.2**). In the focus of neurodegenerative disease the neurodestructive role of activated microglia has been widely accepted, considering microglia as promising target for anti-inflammatory treatment. The exact mechanisms by which activated microglia kill neurons are complex and not fully understood, but appear to involve expression of NADPH oxidase (NOX), inducible NO synthase (iNOS) and microglial phagocytosis of neurons[61]. Nevertheless, recent work also highlights neuroprotective aspects of microglial activation[62,63], including secretion of protective cytokines (TGF-β, IL-10) or increased phagocytosis of debris[64]. This thesis focuses on potentially beneficial aspects of microglial activation by investigating the regulation of microglia antigen presenting capacities that balance between inflammatory (pathogenic) or anti-inflammatory (protective) T cell responses.

1.4 Microglia – $CD4^+$ T cell interaction

This thesis aims to understand the interaction of microglia and $CD4^+$ T cells. Therefore the activation by antigen, formation of distinct lineage and classification of the latter should be briefly explained at this point.

Activation of naive $CD4^+$ T lymphocytes occurs in secondary lymphoid organs (lymph nodes, spleen, Peyer's patches, tonsils) during their recirculation. Activation of naive $CD4^+$ T lymphocytes requires recognition of their specific antigen bound to MHCII molecules by the T cell receptor (TCR - MHCII-Ag, signal 1) and co-stimulators, such as B7-1 and B7-2 that are expressed on activated antigen-presenting cells (signal 2). Signals 1 and 2 are provided by professional antigen presenting cells (APC), such as dendritic cells, macrophages and B cells, which efficiently internalize antigen, migrate to secondary lymphoid organs and present antigens via MHCII molecules. Signal 3, the polarization signal, is mediated by soluble and membrane bound factors, such as interleukines (IL-12, IL-4) or chemokine ligands (e.g. CCL2)[65]. Th1 cells differentiate in the presence of IL-12, produce mainly IFNγ and IL-2, and are involved in cell-mediated immunity. Th2 cells develop in the presence of IL-4, secrete primarily IL-4, IL-5 and IL-13 and contribute to humoral immunity. On the transcriptional level T-bet and STAT4 transcription factors promote Th1 cell fate, whereas GATA3 and STAT6 govern Th2 cell polarization. Upon activation, effector T cells migrate to any site of infection or inflammation, where they re-encounter their specific antigen.

Like naive $CD4^+$ T cells, naturally arising $CD4^+$ regulatory T cells (nTreg) emerge from the thymus, express the transcription factor Foxp3, and are engaged in suppressing immune responses towards self and non-self antigens. Unlike the majority of thymus-derived naive $CD4^+$ T cells, nTreg cells are already functionally mature and antigen-primed[66]. Naive $CD4^+$ T cells can also differentiate into $Foxp3^+$ regulatory T cells in peripheral lymphoid tissues, referred to as inducible Treg (iTreg)[67]. The Th17 lineage was initially identified based on the discovery of IL-23 as a strong inducer of IL-17 producing cells. Th17 cells mainly secrete IL-17A, IL-17F, IL-21 and IL-22. Th17 cells differentiate from naive $CD4^+$ precursors in the presence of TGF-β and IL-6 or IL-21 driven by the master transcription factor RORγt[68,69].
T lymphocytes migrate into the CNS during health and disease[12]. Under steady-state conditions very few T cells were found to cross the BBB but could be detected within the brain parenchyma[70]. Very little is known about the phenotype those cells acquire after having penetrated the BBB and what cells are engaged in their re-activation. During pathological conditions such as infections, ischemia or inflammatory diseases (e.g. MS), leukocytes are found to traffic into the CNS parenchyma[13]. Under the same pathological conditions, microglia can up-regulate a distinct set of molecules needed for antigen presentation, like MHCII, CD40 or CD86. *In vitro* studies clearly showed antigen presenting capacities of microglia[71,72], however, it is still debated whether microglia present myelin antigens to autoreactive T cells *in vivo* thereby potentially contributing to MS pathology. Indirect evidence is provided by experiments using mice depleted of microglia (CD11b-HSVTK) that showed repressed development of EAE disease[49]. Other studies demonstrated that their relative capacity in priming $CD4^+$ T cells is lower than that of professional APC, such as DCs[72–74]. Microglia isolated *ex vivo* from the brain of EAE mice were shown to efficiently present exogenous and endogenous CNS antigens[75]. Because microglial cells are able to migrate to sites of CNS injury, up-regulate APC molecules and secrete pro-inflammatory as well as anti-inflammatory cytokines, their contribution in regulating $CD4^+$ T cell responses is quite possible.

1.5 Regulatory T cells

Ensuring immune homeostasis and maintaining immunological self-tolerance are the main tasks of $CD4^+$ regulatory T cells (Treg). The term “regulatory” hereby refers to its ability to transfer immune unresponsiveness *in vivo* from one animal to another[76]. Disturbances in Treg function or numbers have been implicated in many autoimmune diseases, such as multiple sclerosis, rheumatoide arthritis, Crohn's disease or autoimmune liver disease. Regulatory T cells can be developmentally classified into natural, thymus-derived Treg (nTreg) and peripherally induced Treg (iTreg), both exhibiting suppressive activity in order to control pathological and physiological immune responses[77]. Different subsets of peripherally

induced Treg were identified, such as IL-10-secreting Tr1 cells[78] or TGFβ-secreting Th3 cells[79], whose participation in the physiological control of adaptive immune responses and self-tolerance need to be further clarified.

Most Treg cells constitutively express CD25[77], the α chain of the IL-2 receptor, and therefore CD25 has been used for long to characterize Treg cells. However, CD25 expression is not exclusive for Treg cells, but also apparent on non-regulatory activated $CD4^+$ T cells. In addition to CD25 further characterization revealed that the expression of other surface markers such as glucocorticoid-induced tumour necrosis factor receptor (GITR) or cytotoxic T-lymphocyte antigen-4 (CTLA-4) are also implicated in regulatory function[80–82], but their expression is also seen in other activated $CD4^+$ T cells. Finally, the Foxp3 transcription factor forkhead box P3 was identified as being the master regulator for development and function of regulatory T cells[83,84]. A defect in *foxp3* gene expression was first found in the Scurfy mouse[85], an x-linked recessive mutant strain, that is lethal in hemizygous males one month after birth and characterized by overproliferation of $CD4^+$ T cells and overproduction of numerous, proinflammatory cytokines[86]. In humans, several mutations within the *foxp3* gene had been linked to the X-linked syndrome of immune dysregulation, polyendocrinopathy, enteropathy (IPEX), characterized by an early onset of multiorgan autoimmunity[87]. The clinical and immunological similarities between IPEX patients and the Scurfy mouse lead researchers to suggest that *foxp3* deficiency results primarily in the lack of functional regulatory T cells, rather than in the failure to repress inflammatory cytokine expression[88]. In line with that, further experiments demonstrated that retroviral transduction of *foxp3* converted naive $CD4^+$ T cells into functionally suppressive T cells in mice[89]. To facilitate studies of Treg development and function, *Foxp3*^{EGFP} reporter mice were generated[90,91]. With this approach, several groups also demonstrated a developmental relationship between Th17 and peripherally induced Treg lineages[91,92]. Upon antigen-specific activation, $CD4^+$ T cells can transiently co-express Foxp3 and RORγt in the presence of TGF-β[93] and differentiate depending on dominance of IL-6 or retinoic acid into Th17 or $Foxp3^+$ regulatory T cells, respectively[92]. Together, Foxp3 resembles not only a Treg-specific marker, but also a lineage commitment factor for regulatory T cells in mice. However, there are differences between humans and mice particularly regarding the regulatory phenotype of $Foxp3^+$ T cells. As with mice, natural occurring and peripherally induced human regulatory T cells both express the Foxp3 transcription factor. However, recent studies demonstrated that Foxp3 expression can be induced in human $CD4^+$ T cells by e.g. TCR-mediated activation without any suppressive activity of resulting $Foxp3^+$ cells[94,95].

Several models exist by which regulatory T cells can mediate suppression and modulation of immune cells. One line of evidence suggests that suppression requires direct cell contact between Treg and effector T cells thereby inhibiting effector T cell proliferation[96]. Other

studies proved that immunosuppressive IL-10 secreted by Treg cells mediates tolerance[97,98]. Likewise, in a model of airway tolerance induced by a low dose of inhaled antigen, membrane-bound TGF-β on Foxp3$^+$ Treg cells was shown to be important for their immunosuppressive ability in a cell contact-dependent way[99]. In another scenario, regulatory T cells act by, e.g. down-regulating CD40, CD80, and CD86 co-stimulatory molecules on DCs[100], thereby interfering with T cell activation[101]. Most likely, CD4$^+$CD25$^+$Foxp3$^+$ Treg suppressor mechanisms involve a combination of all, cytokine release, cell-cell contact and APC modulation.

1.5.1 Induction of regulatory T cells in the periphery

Upon identification of the fundamental role of regulatory T cells in immune homeostasis, allograft rejection, inhibition of autoimmune phenomena and allergic reactions[82], much effort has gone into developing protocols to expand or induce alloreactive or antigen-specific Treg cells for therapy *in vivo* or *in vitro*. A few examples for induction of regulatory T cells from naive CD4$^+$ precursors in the periphery are given below.

The oral administration of low-dose antigen is the most prominent way of tolerance induction *in vivo*[102]. This so called mucosal tolerance is mediated by Th2, Th3 and Foxp3$^+$ Treg cells and was applied to numerous autoimmune disease models, including EAE, uveitis and arthritis, plus non-autoimmune diseases such as asthma, atherosclerosis, graft rejection, allergy, colitis, stroke, and models of Alzheimer's disease[79,102,103]. In an early study, the oral administration of myelin basic protein (MBP) demonstrated EAE suppression in SJL mice mediated by peripheral tolerance induction. T cells isolated from these tolerized mice produced predominantly TGF-β, IL-10 and IL-4[104]. In turn, high doses of antigen together with a strong activation of DCs lead to increased T cell proliferation rather than conversion of Foxp3$^+$ Treg cells in a subcutaneous immunization set up designed for studying the extrathymic of naive T cells[105]. In that study, the idea of “subimmunogenic” DC stimulation came up, which describes the combination of low-dose antigen and the lack of co-stimulation. Antigen-unspecific induction of Foxp3$^+$ Treg cells was observed, when glatiramer acetate (GA, Copaxone), a drug approved for MS therapy, was administered subcutaneously[106]. GA treatment induced anti-inflammatory type II monocytes characterized by increased IL-10 and TGF-β secretion, reduced co-stimulatory molecule expression and reduced STAT-1 phosphorylation upon stimulation. On top, when adoptively transferred GA-treated type II monocytes reversed established EAE[106].

In addition to *in vivo* Treg conversion, much of the research is focused on generating antigen-specific or polyclonal Treg cells for adoptive immunotherapy *in vitro*. Methods used for antigen-specific expansion include the repetitive stimulation of Treg exhibiting antigen specificity against an allopeptide with suboptimal peptide-primed DCs[107]. Repetitive

stimulation with anti-CD3/CD28 coated Xcyte beads[108] or anti-CD3/CD28 stimulation in the presence of artificial APC ($CD32^+$ L cells)[109] was reported for polyclonal Treg expansion. Using Xcyte beads, the authors report on 200-fold expansion of human Treg, whereas artificial APC led to an average 13.000-fold expansion within 3-4 weeks, while maintaining suppressive activity. All approaches require high doses of exogenous IL-2 to induce proliferation.

1.5.2 Regulatory T cells in the CNS

Although many studies examined the role of regulatory T cells in autoimmune and also neurodegenerative disease settings, such as EAE or stroke[110–112], only a few addressed the presence of Treg cells directly in the CNS. First evidence came from studies with IL-10 deficient mice, which developed a more severe EAE compared to wild-type mice[110–112]. This was supported by finding high levels of IL-10 in the CNS during recovery phase in a model of relapsing-remitting EAE[86]. Moreover, the adoptive transfer of regulatory T cells, as IL-10 producers and suppressors of autoimmune responses, conferred protection from EAE[114]. In addition, the accumulation of Foxp3-expressing regulatory T cells directly in the CNS was shown to correlate with EAE recovery phase, whereas depletion of $CD25^+$ cells inhibited recovery[82]. In turn, more recent data suggested that natural autoantigen-specific Treg cells accumulate in the CNS but fail to control ongoing autoimmune inflammation[110] in chronic progressive EAE. This highlights how little is known about the exact mechanisms by which regulatory T cells contribute to attenuating EAE pathology and the question remains, whether regulatory T cells prevent trafficking of autoreactive Th1 cells into the CNS or are themselves targeted to the CNS[111].

Another line of evidence came from studies in brain ischemia. One report suggested a role for IL-10 secreting regulatory T cells in the functional outcome after acute experimental stroke (MCAO)[111]. Besides demonstrating increased infarct size, pro-inflammatory cytokine expression and enhanced microglia activation after depletion of Treg cells, this report proved that $Foxp3^+$ Treg cells start infiltrating into the ipsilateral hemisphere 3 days after MCAO[112]. Another group used animals tolerized to myelin-antigens before experimental stroke to show that TGF-β-secreting T cells in the brain mediate neuroprotection[112]. In summary, the presence of regulatory T cells in the CNS and their contribution to local immune responses in CNS autoimmune disease or secondary inflammation after stroke or brain injury is not fully understood.

1.6 Aim of the Study

Previously, our lab reported on how brain infiltrating effector T cells affect microglial expression of co-stimulatory molecules depending on their polarization (Th1 versus Th2)[118]. In a next study, our group demonstrated reduced EAE severity in mice subjected to entorhinal cortex lesion, suggesting a long-term tolerogenic effect induced by fiber tract injury[119]. To further clarify the mechanism of long-term tolerance towards brain antigens we examined the role of regulatory T cells. The central question of this thesis was whether and to what extend microglia cells can directly induce a regulatory T cell response.

We hypothesized that microglia activation is a fine-tuned mechanism contributing to immune regulation in the CNS. In this thesis the $CD4^+$ T cell interactions with microglia were studied regarding

(I) $CD4^+$ T cell activation/differentiation during co-culture,

(II) characterization of microglial phenotypes and

(III) potential *in vivo* implications of MHCII expression within the CNS.

2 Materials and Methods

2.1 Materials

2.1.1 Mice

C57Bl6/J wild-type mice were purchased from Elevage Janvier (Le Genest St. Isle, France). Foxp3^{EGFP} reporter mice (on C57Bl6/J background) were provided by B. Malissen (CIML, France) and have been described previously[90]. T cell receptor transgenic C57BL/6-Tg(Tcra2D2, Tcrb2D2)1Kuch/J mice, commonly known as 2D2 mice, C57Bl6/J-Thy1.1 congenic mice and Major Histocompatibility Complex Class II (MHCII) deficient mice (B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J) were purchased from The Jackson Laboratory (Maine, USA). The 2D2 mice, reactive to myelin oligodendrocyte glycoprotein ($MOG_{35–55}$), were intercrossed with Foxp3^{EGFP} reporter mice to obtain 2D2.Foxp3^{EGFP} mice (**Fig. 2.1**). IL-10 deficient (IL-10$^{-/-}$) mice on C57Bl6/J background were provided by A. Hamann (DRFZ, Germany). All mice were bred and maintained under specific pathogen-free conditions at the animal facility of Charité - Universitätsmedizin Berlin.

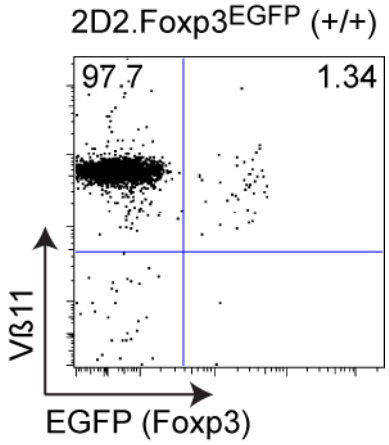

Fig. 2.1 Vβ11 profile of CD4$^+$ T cells from 2D2.Foxp3^{EGFP} transgenic mice.
Blood samples of 6 weeks old mice were analyzed for the expression of MOG-specific TCR (Vβ11) and Foxp3^{EGFP} by flow cytometry.

2.1.2 Kits

Kit System	Manufacturer
Anti-Mouse/Rat Foxp3 Staining Set PE	eBioscience
BD Cytofix/Cytoperm™	BD
DNeasy Blood Kit	QIAGEN
FlowCytomix™ Basic Kit	eBioscience
Mouse $CD4^+CD25^+$ Regulatory T Cell Isolation Kit	Miltenyi Biotec
Mouse $CD4^+$ T Cell Isolation Kit II	Miltenyi Biotec
Mouse CD90.2 MicroBeads	Miltenyi Biotec
Mouse IL-10 Platinum ELISA	eBioscience
Mouse IL-10/ TNFα /IL-4 / IL-23/ MCP-1/ IL-17/ IL-6/ IL-13/ IFNγ FlowCytomix Simplex Kit	eBioscience
Mouse IL-17 Secretion Assay – Detection Kit	Miltenyi Biotec
Mouse IFNγ Secretion Assay – Detection Kit	Miltenyi Biotec
QuantiTect Rev. Transcription Kit	QIAGEN
RNeasy Mini Kit	QIAGEN
TaqMan® Fast Universal PCR Master Mix	Applied Biosystems

Table 2.1 Kits.
All Kits were applied according to the manufacturer's instructions.

2.1.3 Buffers and media

Name	Content
DMEM	10% FCS, 1% Pen/Strep, 50 µM β-Me
FACS buffer	2% FCS and 2 mM EDTA in PBS
HBSS	w/o and w Ca^{2+} and Mg^{2+}
L929 supernatant	media obtained by culturing confluent L929 mouse fibroblasts for 14 days
Red blood cell lysis buffer	9:1 mixture of 0.16 M NH_4Cl, pH. 7.2, and 0.17 M Tris base, pH 7.2
RPMI-1640	10% FCS, 1% Pen/Strep, 50 µM β-Me

Table 2.2 Buffers and media.

2.1.4 List of monoclonal antibodies

Antigen	Clone	Manufacturer
CD3ε	145-2C11	BD
CD4	RM4-5	BD
CD8	53-6.7	BD
CD11b	M1/70	BD
CD11c	N418	eBioscience
CD25	PC61	BD
CD28	37.51	BD
CD45	30-F11	BD
CD45R/B220	RA3-6B2	BD
CD154	MR1	BD
Foxp3	FJK-16s	eBioscience
IFNγ	AN18.17.24	Miltenyi
IL-4	11B11	BD
IL-17	TC11-18H10	Miltenyi
IL-17a	MM17F3	eBioscience
MHC II	M5/114.15.2	BD
TCR β chain	H57-597	BD
Vα3.2	RR3-16	BD
Vβ11	RR3-15	BD

Table 2.3 List of monoclonal antibodies.

2.1.5 List of pre-developed gene expression assays

Assay ID	Gene symbol	Gene name
Mm00439614_m1	IL-10	interleukin 10
Mm00441724_m1	TGF-β	transforming growth factor, beta 1
Mm00441891_m1	CD40	CD40 antigen
Mm00444543_m1	CD86	CD86 antigen
Mm00445235_m1	Cxcl10	chemokine (C-X-C motif) ligand 10
Mm00482914_m1	CIITA	class II transactivator

Assay ID	Gene symbol	Gene name
Mm00492586_m1	IDO	indoleamine 2,3 dioxygenase 1
Mm00711662_m1	CD80	CD80 antigen
Mm01261022_m1	Rorc	RAR-related orphan receptor gamma
Mm01309902_m1	iNOS	nitric oxide synthase 2, inducible
Mm01545399_m1	HPRT	hypoxanthine guanine phosphoribosyl transferase

Table 2.4 List of pre-developed gene expression assays.

2.1.6 List of cytokines

Cytokine	Manufacturer
GMCSF	R&D Systems®
IFNγ	Peprotech
IL-2	R&D Systems®
IL-10	R&D Systems®
IL-12	R&D Systems®
MCSF	Peprotech

Table 2.5 List of cytokines.

2.1.7 List of reagents

Reagent	Manufacturer
Baytril	Bayer
Brefeldin A	Sigma-Aldrich
Cell proliferation dye eFluor® 670	eBioscience
Clodronate	Calbiochem
Collagenase VIII	Sigma
DNase I	Roche
DMEM	Gibco
EDTA	Invitrogen
FCS (s 0115/Ch:1073 S)	Biochrome
Freunds Complete Adjuvant	Difco Laboratories/ BD
Glucose	B. Braun

Reagent	Manufacturer
HBSS w/o and w Ca2+, Mg2+	Gibco
Histopaque 1083	Sigma
Ionomycin	Cell Signaling Technology
Ketamine	Sigma-Aldrich
MBP	Biosynthan
MOG	Pepceuticals
Mycobacterium tuberculosis H37 Ra	Difco Laboratories/ BD
Penicillin/Streptomycin	Invitrogen
Percoll	Biochrome AG
Pertussis toxin	List Biological Laboratories
Rompun	Bayer
Poly-D-Lysine	Millipore
RPMI-1640	Gibco
SNARF-1	Molecular Probes
Trypsin-EDTA	Gibco
2-Mercaptoethanol	Gibco

Table 2.6 List of reagents.

2.1.8 List of consumable material

Consumables	Manufacturer
Cell strainer, 40 µm and 70 µm	BD
Cell culture flasks 75 cm2	BD
Cell Separation Columns MS, LS, LD	Miltenyi Biotec
Combitips 10 ml	Eppendorf
Conical Centrifuge Tubes 15 ml, 50 ml	BD
Filter Tips 2.5 - 1000 µl	Sarstedt
Microplates and multi-well plates	BD
MiniCollect EDTA Tubes 0.5 ml	Greiner Bio-One
Needles	B. Braun Melsungen AG
PCR SoftTubes 0.2 ml	Biozym Diagnostik GmbH

Consumables	Manufacturer
Pipettes 2.5 – 25 ml	BD
Round bottom test tubes	BD
Safe Lock Tubes 0.5 - 2 ml	Eppendorf
Syringes	BD
3-Way Stopcock	B. Braun Melsungen AG

Table 2.7 List of consumable material.

2.1.9 List of Instrumentation

Instruments	Manufacturer
Axiovert 135	Zeiss
Centrifuge 5810	Eppendorf
ELISA Reader/ Washer	Tecan
FACSAria	BD
FACSCanto II	BD
Incubator	Binder
LSR II	BD
Microcentrifuge 5415R	Eppendorf
7500 Real Time PCR System	Applied Biosystems

Table 2.8 List of general equipment.

2.2 Animal models

2.2.1 Entorhinal cortex lesion (ECL) and isolation of CNS infiltrating immune cells

ECL involves the stereotactical lesion of the perforant path (**Fig. 2.2**), a myelinated fiber tract connecting the entorhinal cortex with the hippocampus, leading to anterograde degeneration of the hippocampus[54].

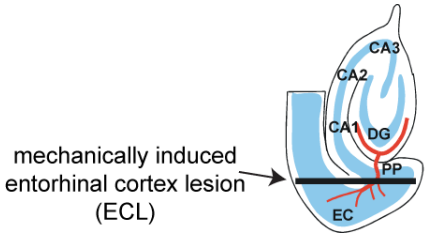

Fig. 2.2 Entorhinal cortex lesion (ECL) model.
EC= Entorhinal Cortex, DG= Dendate Gyrus, PP= Perforant Path, CA1-3= Cornu Ammonis (adapted from Ch. Brandt)

Stereotaxic surgery (ECL) was performed as previously described[120]. In brief, mice were anesthetized using a ketamin cocktail (20% Ketamin and 8% Rompun® in 0.9% NaCl; 0.01 ml/g body weight). After exposing the skull, a burr-hole was drilled and the left entorhinal cortex was lesioned with a 2 mm scalpel adjusted to the following coordinates from Lambda: anterior-posterior 0.3 mm, lateral 1.8 mm and vertical down to the base of the skull. For sham operation, animals were anesthetized and the scalp incised. However, instead of opening the skull, the scalp was sutured back and animals were allowed to recover. For flow cytometric analysis 14 days later, blood samples were drawn from ECL operated animals before they were transcardially perfused with saline, and both hemispheres, lymph nodes and spleens were collected. From blood samples, lymphocytes were separated using Histopaque 1083 (Sigma). Single cell suspensions from lymph nodes and spleen were prepared by forcing the tissues through a cell strainer (pore size 70 µm). For flow cytometric analysis of brain infiltrating cells, 1/3 of lesioned posterior ipsi- or contralateral hemisphere was incubated for 30 min at 37 °C with Collagenase VIII (Sigma) for dissociation. The tissue was squeezed through a 70 µm cell strainer and mononuclear cells were separated using Percoll (Biochrome AG) gradient centrifugation (30 min) with a gradient of 35% and 70%. Cell suspensions from brain, lymph nodes, spleen and blood were stained for FACS analysis (FACSCanto II, BD).

2.2.2 Mixed bone marrow chimeras

Mixed bone marrow chimeras were generated as previously described[121]. Bone marrow from the femurs of male C57Bl6/J wild-type (WT) and MHCII deficient ($MHCII^{-/-}$) and $Foxp3^{EGFP}$

mice was isolated and depleted of $CD90.2^+$ cells using anti-CD90.2 beads (Miltenyi Biotec). WT, $MHCII^{-/-}$ or $Foxp3^{EGFP}$ bone marrow (8×10^6 cells) was administered intravenously into respective recipient mice after lethal irradiation (1100 cGy) given as a split dose. Mice were kept on antibiotics (0.001% Baytril® (Bayer) in drinking water) for 6-8 weeks after reconstitution until they were subjected to blood phenotyping. On the day before sterotaxic surgery (ECL) all chimeric mice received 2×10^7 purified $CD4^+$ T cells (CD4 T cell isolation kit, Miltenyi Biotec) from $Foxp3^{EGFP}$ reporter mice.

2.2.3 Experimental autoimmune encephalomyelitis (EAE)

Female C57Bl6/J mice were immunized s.c. at four sites on the flank with 250 µg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGGK, Pepceuticals, UK) emulsified in complete Freund's adjuvant containing H37 RA (BD Bioscience). On the day of immunization and two days later, pertussis toxin (400 ng, List Biological Labs, CA) was administered i.p. Mice were observed for clinical signs of EAE (paralysis progressing from tail to head) for up to 30 days and scored daily using the following scale: (0) no disease, (1) limp tail and/or hind limb ataxia, (2) hind limb paresis, (3) hind limb paralysis, (4) hind limb and fore limb paralysis, and (5) death.

2.3 Cell biology protocols

2.3.1 Generation of Th1 cells

We generated Th1 cells as previously described[122]. $CD4^+$ T cells were isolated from lymph nodes and spleens of C57Bl6/J mice using $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec). Cells were seeded at a density of 1×10^6/ml in complete RPMI-1640 and stimulated with a cocktail of 5 µg/ml anti-CD3ε, 5 µg/ml anti-CD28, 1 µg/ml anti-IL-4 and 1 ng/ml IL-12. Two days later IL-2 was added to final concentration of 1 ng/ml. Cells and cell supernatant were harvested on day 4-5 after preparation.

2.3.2 Generation of bone marrow derived macrophages and dendritic cells

Bone marrow was isolated from the femur, tibia and humerus of adult C57Bl6/J mice by cutting off bone ends and flushing it out using a syringe. The cell suspension was filtered (70 µm cell strainer) to remove any bone and tissue fragments and centrifuged for 5 min at 1200 rpm. Erythrocytes were lysed in Tris-NH_4Cl solution. Bone marrow cells were seeded at a density of 7.5×10^5/ml in complete RPMI-1640 and stimulated with 2 ng/ml MCSF (Peprotech) to be differentiated into macrophages and 10 ng/ml GMCSF (Peprotech) for bone marrow derived dendritic cells. After 6 days of culture, adherent cells were harvested and analyzed by flow cytometry (FACSCanto II, BD) for CD11b, CD11c and MHCII.

2.3.3 Generation of primary microglia

Mixed glial cell cultures were prepared from cerebral cortex of newborn (P0-2) mice as described previously[123]. In brief, cortices and brain were obtained and the bloodvessels and meninges were carefully removed. Tissues were dissected and subjected to enzymatic dissociation (25 µg/ml DNase I (Roche) in 0.05% Trypsin-EDTA (Invitrogen), 10 min at 37°C), washed in FCS containing HBSS and resuspended in complete DMEM. Mixed glial cells were cultured in poly-D-lysine-coated (Sigma) flasks at 37°C and 5% CO_2. The day after preparation, cell debris was removed and the media replaced. After 10-12 days, microglia were separated from the underlying astrocytic layer by gentle shaking of the flask. Cultures usually contained > 95% microglial ($CD11b^+$) cells, as determined by flow cytometry (FACSCanto II, BD).

2.3.4 Generation of adult microglia

Mixed glial cell cultures were prepared as described above. On the second day after preparation, mixed glial cultures were stimulated with L929 cell conditioned media. Another two days later, the bisphosphonate clodronate (200 µg/ml for 48 h, Calbiochem®) was used to selectively deplete postnatal microglia. Dead cells were removed by gentle agitation of the flask over night. Adult microglia were isolated from cortices, cerebellum and brainstem of male C57Bl6/J mice. The tissue was homogenized and incubated with 2.5% Trypsin (Invitrogen) at 37°C for 10 min. FCS containing media was used to stop enzymatic digestion and cells were pelleted (130 x g, 10 min). DNase I was added to remove DNA and the cell suspension was forced through a 40 µm cell strainer before seeding the adult brain cells onto the postnatal astrocytic layer. After one week of culture in the presence of L929-conditioned media, adult microglial cells were harvested by gentle shaking of the flask.

2.3.5 Microglia – T cell co-culture

Microglia were seeded into 96-well plates (4×10^5/ml) in complete DMEM and stimulated 24 h later with recombinant IFNγ (Peprotech) at final concentrations of 10 U/ml for low-dose and 100 U/ml for high-dose condition, respectively. Another 24 h later, cells were pulsed with myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide (MEVGWYRSPFSRVVHLYRNGGK, Pepceuticals) at final concentrations of 1 µg/ml for low-dose and 10 µg/ml for high-dose stimulation, respectively. Untouched $CD4^+$ T cells were purified from lymph nodes and spleens of 2D2.$Foxp3^{EGFP}$ mice on C57Bl6/J background using $CD4^+$ T cell isolation Kit II (Miltenyi Biotech), if not indicated otherwise. Stimulated microglia cultures were washed, isolated $CD4^+$ T cells were seeded onto the microglial layer (2×10^6/ml) and co-cultured for 7 days (37°C, 5% CO_2). Following co-culture, $CD4^+$ T cells were harvested, stained for CD11b,

CD4 and CD25, and analyzed by flow cytometry (FACSCanto II, BD) for the expression of GFP (Foxp3) and respective cell markers.

For *Kinetic analysis of microglia-mediated T cell response,* $CD4^+$ T cells were harvested and analyzed on a daily base for indicated markers. In addition, supernatants were collected and analyzed for cytokine secretion pattern using FlowCytomix Multiple Analyte Detection System (eBioscience).

For *microglia-mediated de novo generation of Foxp3$^+$ Tregs,* $CD4^+$ T cells were further depleted for CD25 and $Foxp3^{EGFP}$ positive cells by flow cytometry (FACSAria, BD) (purity > 99%). $CD4^+CD25^-Foxp3^-$ cells were then seeded onto the microglial layer and co-cultured for 7 days.

For analyzing *proliferation of microglia-induced Treg cells*, purified $CD4^+$ T cells were incubated in PBS containing 5 µM cell proliferation dye eFluor® 670 (eBioscience) for 10 min at 37°C before co-culturing them with activated microglia. Proliferation was assessed on day 2 (control) and day 6 of co-culture by analyzing dilution of eFluor® 670 using flow cytometry (FACSCanto II, BD).

For analyzing *MHCII dependency in microglia-mediated Tregs induction,* postnatal microglia were prepared from MHCII deficient mice (B6.129S2-*H2*$^{dlAb1\text{-}Ea}$/J) and used for the co-culture assay.

For analyzing *functional activity of microglia-induced Treg in vivo,* $CD4^+CD25^+$ T cells were purified from co-cultures after 7 days using $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotech) and adoptively transferred into female C57Bl6/J recipients one day before they were subjected to immunization of EAE.

2.3.6 T cell suppression assay

SNARF-1 labeled $CD4^+$ responder T cells (3 x 10^5/well) from 2D2 mice were seeded together with irradiated splenic cells (5 x 10^5/well) in a 96-well plate. FACS-sorted (FACSAria, BD) microglia-induced $CD4^+CD25^+Foxp3^+$ Treg cells and responder T cells were co-cultured at various ratios ranging from 1:5 up to 1:100 in the presence of TCR peptide (10 µg/ml $MOG_{35\text{-}55}$) for 4 days (37°C, 5% CO_2). Proliferation of responder T cells was assessed by SNARF-1 dilution (emission at 640 nm) using flow cytometry (FACSCanto II, BD). $Foxp3^{EGFP+}$ T cells were excluded prior to analysis. In control experiments, SNARF-1 labeled responder T cells were co-cultured with FACS-sorted microglia-induced effector T cells ($CD4^+CD25^+Foxp3^-$).

2.3.7 Intracellular cytokine staining

Microglia-induced T cells were harvested from high-dose activated co-cultures and incubated in RPMI-1640 (1 x 10^7 cells/ml) containing 5 µg/ml CD28 (BD), 20 ng/ml PMA (Sigma) and 1 µg/ml ionomycin (Sigma) for 2 h (37°C, 5% CO_2). Brefeldin A (Sigma) was added to final concentration of 1 µg/ml and cell suspensions were incubated for another 2 h. Following that, cells were stained for the surface markers CD4 and CD25 before fixation and permeabilization using Cytofix/Cytoperm™ (BD). Finally, cells were intracellularly stained for IFNγ and IL-17 (Miltenyi Biotec) and analyzed (LSR II, BD).

2.3.8 Blood lymphocyte phenotyping

Blood samples were obtained from the facial vein of mice and transferred into 500 µl EDTA solution (2 mM) and layered onto 500 µl Histopaque 1083 (Sigma). Following 20 min centrifugation (200 x g) the phases were separated with a tight ring of PBMCs in between. The lymphocyte fraction was collected, washed and stained for further flow cytometric analysis.

2.4 Molecular biology protocols

2.4.1 Methylation studies

Microglia-induced Treg cells from low-dose co-cultures and sexmatched nTreg cells from the lymph nodes of C57Bl6/J were purified using $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Miltenyi Biotech). Genomic DNA was isolated using DNeasy Blood Kit (Qiagen) according to the manufacturer's protocol. DNA samples were analyzed for the degree of methylation of individual CpG motifs within the murine *foxp3* locus as previously described[124]. Two overlapping amplicons were analyzed by bisulfite sequencing (Epiontis GmbH, Berlin). Notably, Treg cells purified from co-cultures and lymph nodes were enriched for CD25, not Foxp3, resulting in a $CD4^+Foxp3^+$: $CD4^+Foxp3^-$ ratio of 1:1 (Treg : Teff).

2.4.2 Real-Time PCR (qPCR)

For extraction of total RNA from microglia the cell-lysat was homogenized by QIAshredder spin columns (Qiagen) and RNA was further purified using RNeasy mini spin columns (Qiagen). For cDNA synthesis the QuantiTect reverse transcription kit (Qiagen) was applied. Subsequently, pre-developed TaqMan gene expression assays and TaqMan Fast Mastermix (Applied Biosystems) were used for amplification of mRNA transcripts. *Hprt* served as housekeeping gene. The results for the relative gene expression were calculated using $2^{-\Delta\Delta CT}$-method. All amplification reactions were performed on ABI PRISM 7500 Fast Real Time PCR System (Applied Biosystems).

3 Results

3.1 Results (I) – CD4$^+$ T cell activation/differentiation during co-culture

This part is focused on the T cell response of microglia – T cell co-culture and describes the induction and kinetic of Foxp3$^+$ regulatory T cells, co-culture cytokine pattern, microglia-mediated T cell proliferation and *de novo* induction of Foxp3$^+$ Treg and their suppressive function *in vitro* and *in vivo*.

3.1.1 Microglia induce CD4$^+$Foxp3$^+$ regulatory T cells

To study whether the neuroimmune system has the potential to achieve an alternative activation state similar to that of peripheral macrophages/ type II monocytes, we investigated antigen-presenting properties of microglia and their phenotypic and functional changes leading to beneficial (regulatory T cell) or detrimental (effector) T cell responses *in vitro*.

Since IFNγ is known to promote cell surface expression of MHC class II molecules on microglia[125], we set up an *in vitro* co-culturing system mimicking different microglial activation states by using recombinant IFNγ in combination with the neuronal antigen myelin oligodendrocyte glycoprotein (MOG). We stimulated microglial cells with either low-dose (10 U/ml /1 µg/ml) or high-dose (100 U/ml /10 µg/ml) IFNγ/MOG, to characterize the effect of microglial activation/maturation stages on the CD4$^+$ T cell response. CD4$^+$ T cells were isolated from 2D2 mice intercrossed with Foxp3^{EGFP} reporter mice (2D2.Foxp3^{EGFP}), providing us with MOG-specific CD4$^+$ cells which express green fluorescent protein (GFP) in Foxp3 positive Treg cells.

Interestingly, upon interaction with low-dose primed microglia, naive CD4$^+$ T cells preferentially differentiated into CD25$^+$Foxp3$^+$ regulatory T cells (33 ± 1.9%, **Fig. 3.1 a, b**). In contrast, high-dose activated microglia induced the expansion of CD25$^+$Foxp3$^-$ T effector cells (33.7%, **Fig. 3.1 a**) rather than CD25$^+$Foxp3$^+$ Treg cells (9.5 ± 1.4%, **Fig. 3.1 b**). The plain co-culture with unstimulated microglia (control) did not evoke a Treg or effector T cell response (**Fig. 3.1 a, b**) and resulted in a substantial loss in the number of CD4$^+$ cells. In the same way, pulsing microglia with MOG peptide alone was not sufficient to induce a regulatory or effector CD4$^+$ T cell response (**Fig. 3.1 a, b**). Stimulating microglia with low-dose IFNγ alone partially induced an effector T cell response, but did not result in differentiation of Treg cells (**Fig. 3.1 a, b**). This clearly demonstrates that microglia can directly influence antigen specific CD4$^+$ T cell responses depending on IFNγ and antigen levels confronted with.

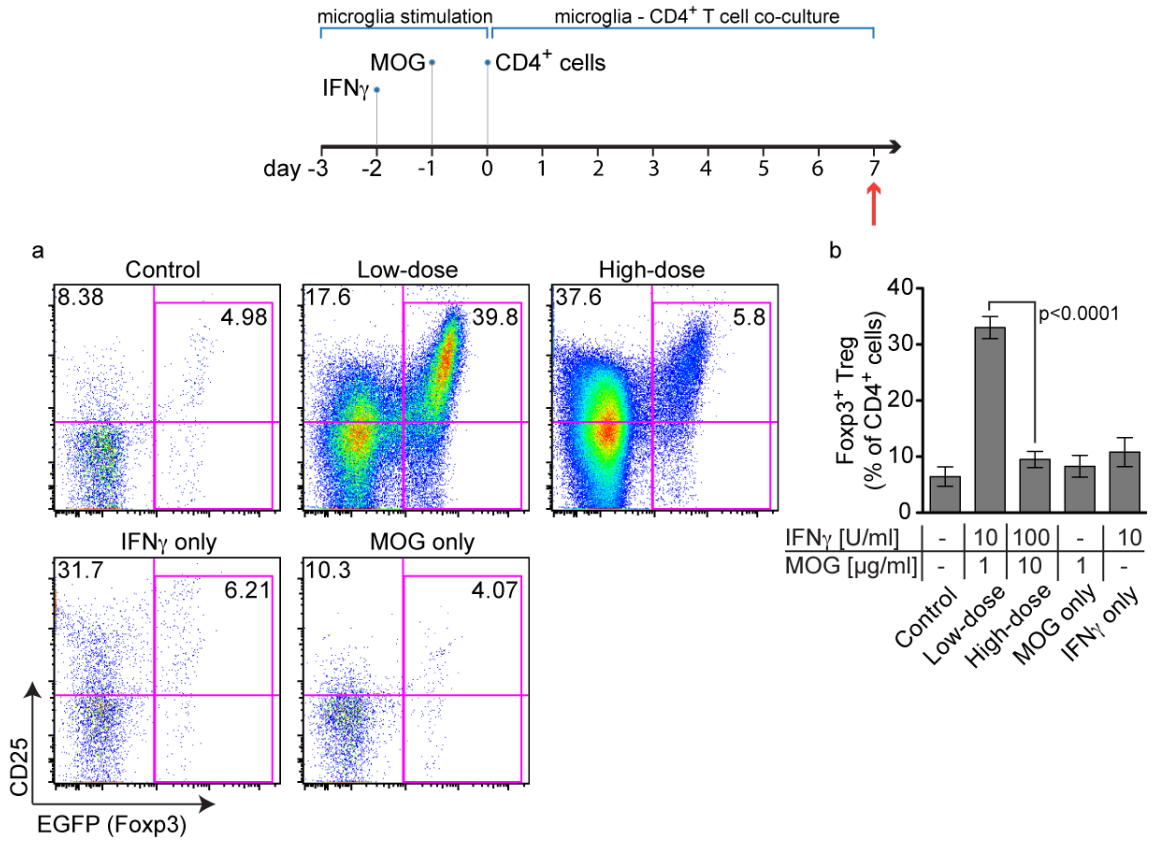

Fig. 3.1 Microglia induce Foxp3$^+$ Treg cells preferentially under low-dose conditions.
Microglia were differentially stimulated and co-cultured with MOG-specific CD4$^+$ T cells from 2D2.Foxp3^{EGFP} mice. Red arrow indicates day of analysis. **(a)** T cells were analyzed by flow cytometry for the expression of Foxp3 and CD25. Density plots are gated for live T cells, CD11b$^-$ and CD4$^+$. Numbers in rectangle gates indicate percent of Foxp3$^+$ cells. Data are representative for n = 4-7 separate experiments, summarized in **(b)** (mean ± SEM, Student's *t* test).

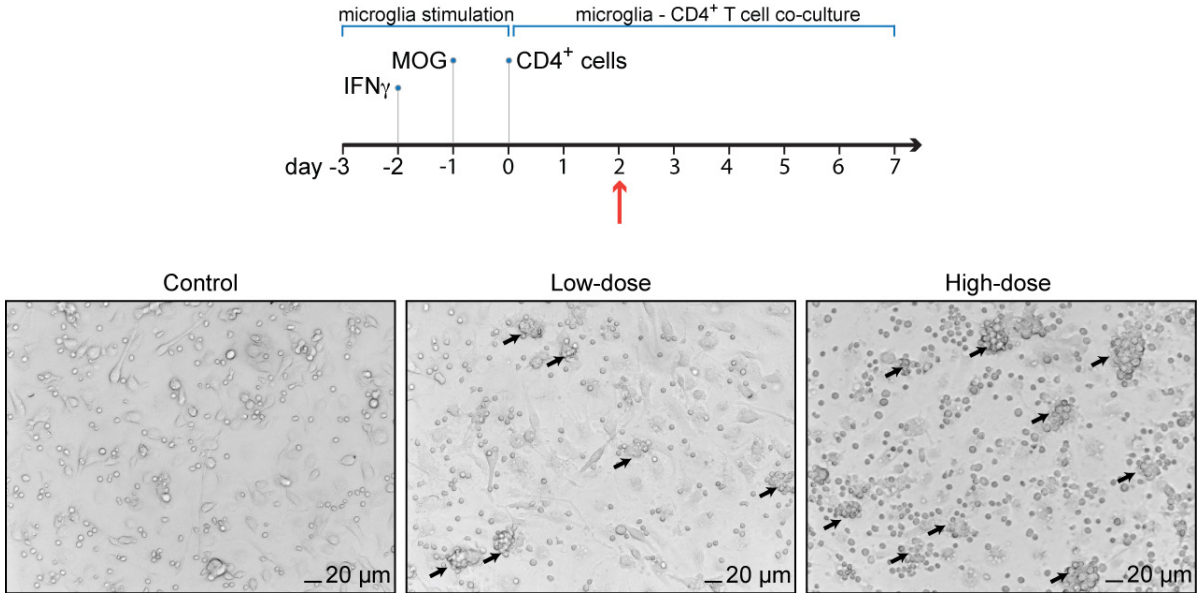

Fig. 3.2 CD4$^+$ T cells are selectively aggregated around activated microglia.
Pictures were taken 48 h after beginning of co-culture using 20 x objective on Axiovert 135 (Zeiss, Germany) microscope and AxioVision Rel. 4.7 (Zeiss, Germany) software. Black arrows indicate T cell-microglia aggregates.

To test, whether the observed T cell response requires physical interaction of T cells and microglia, we monitored the T cell behaviour on microglial cultures. $CD4^+$ T cells formed aggregates around microglia starting 48 h after the beginning of co-culture, but only when microglia had been activated by IFNγ/MOG (**Fig. 3.2**). These findings suggest, that the mechanism of microglia-mediated Treg and effector T cell induction is cell contact-dependent.

3.1.2 Dynamics of microglia-mediated Treg and Teff induction

Kinetic analysis of Foxp3/CD25 expression revealed the first evidence of induction of $Foxp3^+$ cells in low-dose primed co-cultures not earlier than on day 3 (**Fig. 3.3 a, b**), which is 24 h after we observed $CD4^+$ T cells forming aggregates around microglia (**Fig. 3.2**).

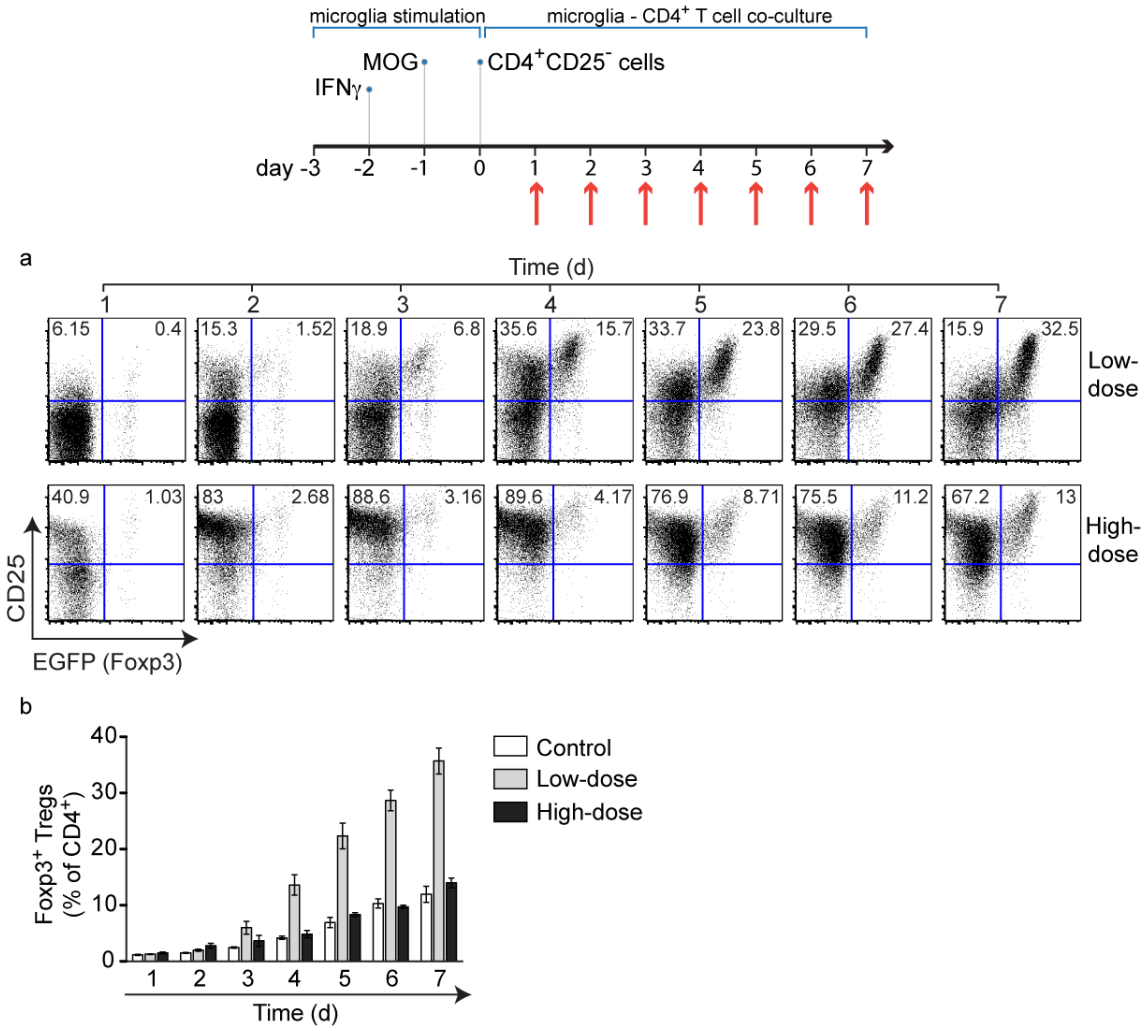

Fig. 3.3 Dynamics of microglia-mediated Treg and Teff induction.
(a) The emergence of $CD4^+CD25^+Foxp3^-$ Teffs and $CD4^+CD25^+Foxp3^+$ Treg cells was analyzed on 7 consecutive days (red arrows) by flow cytometry comparing co-cultures of low- and high-dose activated microglia. Numbers in upper quadrants indicate percent of $CD25^+Foxp3^-$ and $CD25^+Foxp3^+$ cells. Dot plots are representative of n = 3 separate experiments, summarized in **(b)** for GFP^+(Foxp3) Tregs (mean ± SEM).

Notably, even before Foxp3$^+$ Treg cell induction, there is a raising population of CD25$^+$Foxp3$^-$ effector T cells also under low-dose condition (Fig. 3.3 **a**). The percentage of Foxp3 expressing Treg cells constantly increased in low-dose co-cultures over 7 days (**Fig. 3.3 b**). Foxp3 expression was further restricted to the population of CD25 expressing cells (**Fig.** 3.3 **a**). In contrast, high-dose stimulated microglia induced a pool of CD25$^+$Foxp3$^-$ effector cells starting from the first day of co-culture (40.9%) up to 89.6% on day 4 (**Fig.** 3.3 **a**). These two distinct trends of CD4$^+$ T cell response observed after varying IFNγ and MOG concentrations by a factor of 10 suggest a fine-tuned mechanism by which microglia react to IFNγ levels. In addition, finding an activation state in which microglia predominantly induced regulatory T cells raised the question whether microglia induce proliferation or *de novo* generation of Foxp3$^+$ Treg from naive precursors.

3.1.3 Microglia-induced CD4$^+$ T cell response reveals functional plasticity

In order to classify microglia-mediated T cell responses in more detail we determined the cytokines that were produced in microglia-T cell co-cultures using bead-based multiplex immunoassay for flow cytometry (**Fig. 3.4**). Both IFNγ/MOG concentrations substantially increased levels of pro-inflammatory TNFα, with marginal higher TNFα production when stimulated with high-dose IFNγ/MOG and a certain background TNFα level when stimulation was incomplete (IFNγ only). A similar secretion pattern was observed for the chemokine MCP-1, certainly secreted by microglia (**Fig. 3.4**, MCP-1 panel). Over 4 times higher IL-6 levels (630 pg/ml, **Fig. 3.4**, IL-6 panel, day 3) were detected in supernatants of high-dose treated microglial co-cultures versus supernatants from low-dose co-culture (140 pg/ml). In line with the enhanced IL-6 production, we found a massive increase of IL-17 levels (180 pg/ml, **Fig. 3.4**, IL-17 panel, day 2) in high-dose activated co-cultures, pointing towards a Th17 response. Interestingly, the same stimulation condition exclusively induced significant levels of IL-13 (1290 pg/ml, **Fig. 3.4**, IL-13 panel, day 3) and IFNγ (360 pg/ml, **Fig. 3.4**, IFNγ panel, day 1), cytokines known to be secreted during Th2 and Th1 immune responses.

In turn, low-dose stimulation resulted in the highest production of anti-inflammatory IL-10 on day 1-4 of co-culture. Notably, we did not detect any IL-4, IL-1β or IL-23 in our experimental set-up. Regarding the kinetics of secreted cytokines TNFα levels showed only marginal variations over time, whereas MCP-1, IL-6, IL-10 and IL-13 levels increased until day 4 and pro-inflammatory IL-17 and IFNγ decreased from the start of co-culture.

In complementary experiments we used intracellular cytokine staining to further characterize the cells that secrete IL-17 and IFNγ. Interestingly, we identified IFNγ and IL-17 producers to originate from both populations, Foxp3$^+$ Treg and Foxp3$^-$ Teffs (**Fig. 3.5**) Notably, similar frequencies of IFNγ and IL-17 producers were found also in low-dose treated co-cultures

(data not shown), suggesting that high-dose stimulation enhanced the amount of IFNγ and IL-17 on a per cell basis. This cytokine secretion pattern reflects the functional plasticity of microglia induced $CD4^+$ T cell response, leading to secretion of Th1/Th2/Th17 cytokines when stimulated with high-dose IFNγ/MOG or anti-inflammatory IL-10 secretion in response to low-dose stimulation. This data set also indicates that the micromilieu, created by microglial cytokine and chemokine secretion, sensitively responds to the level of IFNγ/MOG.

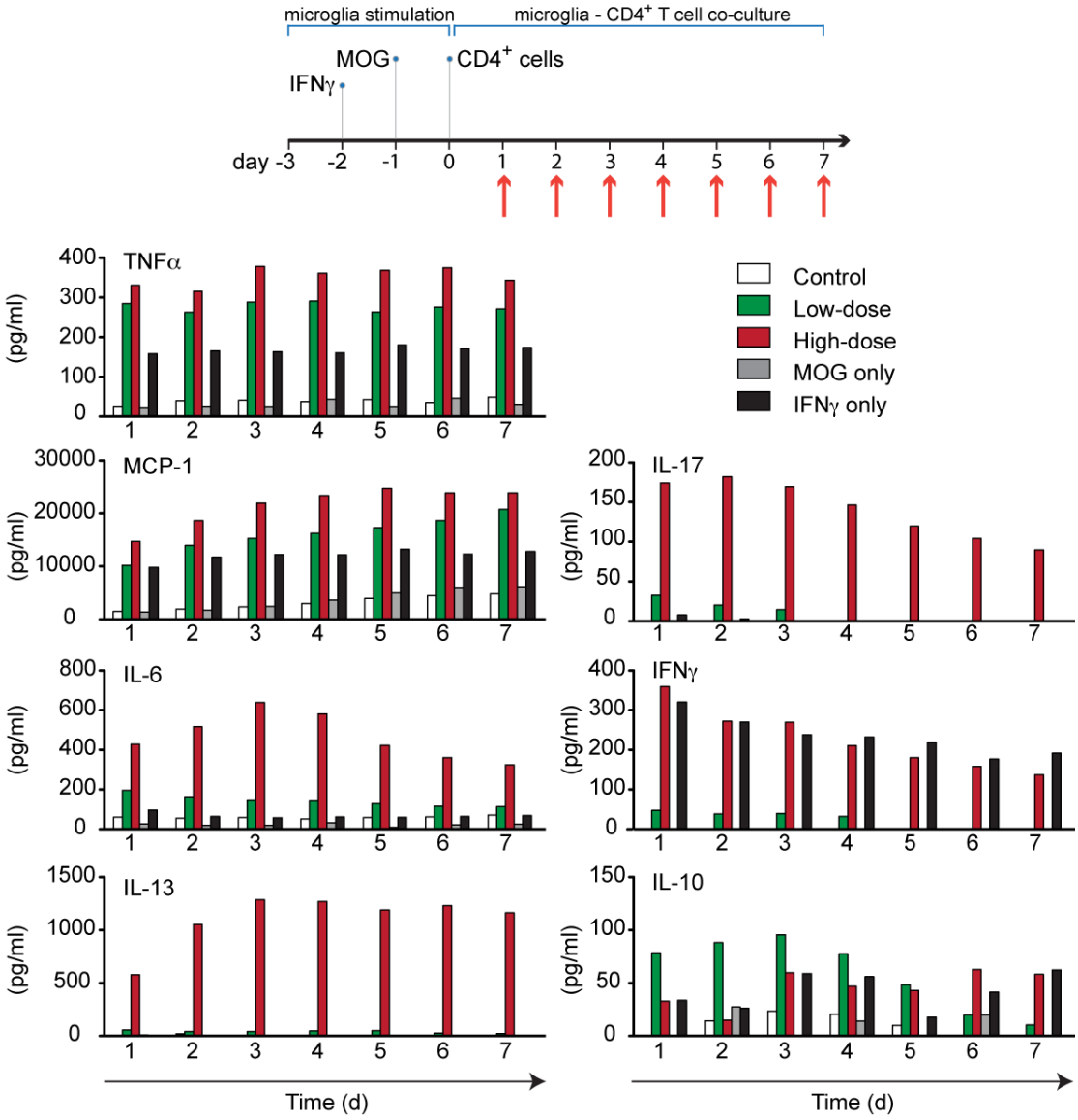

Fig. 3.4 Cytokine secretion pattern of microglia mediated $CD4^+$ T cell responses.
Kinetic analysis of co-culture supernatants using bead-based multiplex immunoassay (eBioscience) for the cytokines TNFα, MCP-1, IL-6, IL-13, IL-17, IFNγ and IL-10. Shown are representative data of three individual experiments.

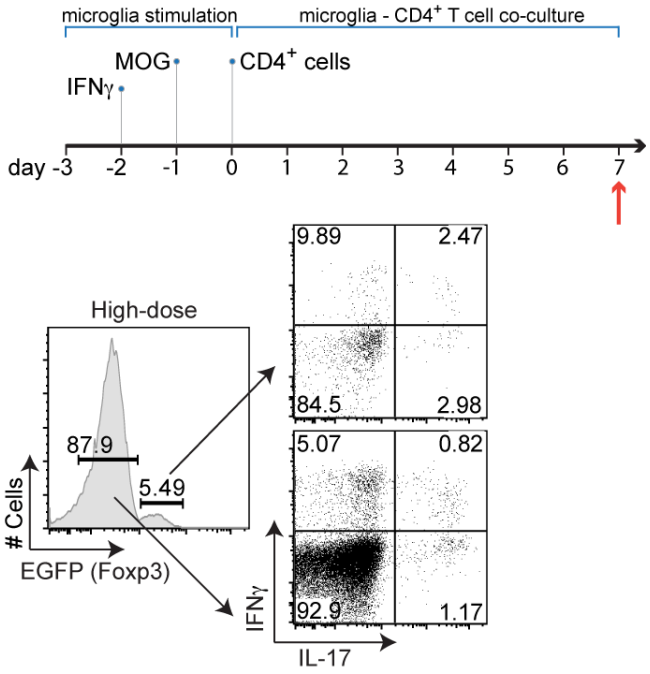

Fig. 3.5 Identification of IFNγ and IL-17 producing cells.
Microglia-induced T cells were harvested, restimulated, fixed, permealized and intracellularly stained with anti-IFNγ-PE and anti-IL-17-APC. Left) Histogram shows T cells derived from high-dose activated co-cultures gated for surface CD11b$^-$ and CD4$^+$. Numbers above bracketed lines indicate percent of Foxp3$^-$ and Foxp3$^+$ population, respectively. Right) Dot plots are showing intracellular cytokine staining for respective Foxp3$^{+/-}$ population. Data are representative of two separate experiments.

3.1.4 Microglia-induced Treg: proliferation versus *de novo* generation

To test, whether the accumulation of Foxp3$^+$ Treg cells during co-culture with activated microglia results from proliferation of pre-existing Treg cells or from the conversion of naive precursors into *de novo* induced Foxp3$^+$ Treg cells, we set up the following experiments. We used CD4$^+$ T cells depleted of CD25$^+$ and Foxp3$^+$ cells (CD4$^+$CD25$^-$Foxp3$^-$ T cells, purity 99%) for co-culture with activated and non-activated microglia. Co-culture with control microglia showed no induction of Foxp3$^+$ cells (1.02%), whereas upon stimulation microglia induced *de novo* Foxp3$^+$ regulatory T cells from naive CD4$^+$ T cells. Under low-dose conditions 19.4% Foxp3$^+$ Treg cells were induced, whereas high-dose stimulation of microglia resulted in only 10.4% *de novo* induced Foxp3$^+$ Treg cells (**Fig. 3.6**). These findings indicate that activated microglia promote *de novo* induction of Foxp3$^+$ regulatory T cells, preferentially when stimulated with low doses of IFNγ and MOG peptide. Notably, compared to total CD4$^+$ cells (Fig. 3.1), *de novo* induced Foxp3$^+$ Treg frequencies from low-dose cultures were reduced. Therefore, we set up a second set of experiments to assess proliferation of Foxp3$^+$ Treg cells.

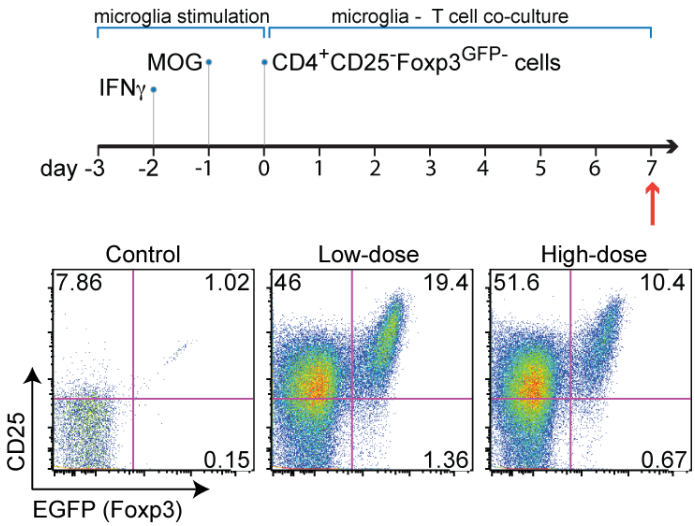

Fig. 3.6 *De novo* induction of Foxp3$^+$ Treg cells after co-culture with microglia.
Flow cytometric analysis of Foxp3$^+$ expressing cells from co-culture of CD4$^+$CD25$^-$Foxp3$^-$ cells with control, low-dose and high-dose activated microglia. Red arrow indicates day of analysis. Numbers in upper right quadrants indicate percent of CD25$^+$Foxp3$^+$ cells. Data are representative of two individual experiments.

Purified CD4$^+$ T cells were labeled with the cell proliferation dye eFluor® 670 (eBioscience) before co-culture with microglia and analyzed for proliferation of Foxp3$^+$ and Foxp3$^-$ cells after six days by flow cytometry. Fluorescent intensity of proliferation dye on day 2 was used as a baseline (**Fig. 3.7**). Co-culture experiments with labeled CD4$^+$ T cells clearly showed proliferation of Foxp3$^+$ Treg cells under both conditions, low-dose (17%) and high-dose (74%) stimulated microglia, whereas under control conditions T cells (Foxp3$^-$/Foxp3$^+$) did not divide (**Fig. 3.7**). Notably, under low-dose conditions Foxp3$^-$ cells showed less proliferation indicating a preferential induction of proliferation in Foxp3$^+$ cells. In contrast, we found 70% of Foxp3$^-$ cells undergoing proliferation in high-dose stimulated microglia cultures.
Thus, we conclude that the enhanced emergence of Foxp3$^+$ Treg cells after co-culture with low-dose activated microglia can be explained as the sum of both, *de novo* induction of Foxp3$^+$ Treg from naive CD4$^+$ T cells as well as Treg proliferation in response to IFNγ/MOG induced tolerogenic signals.

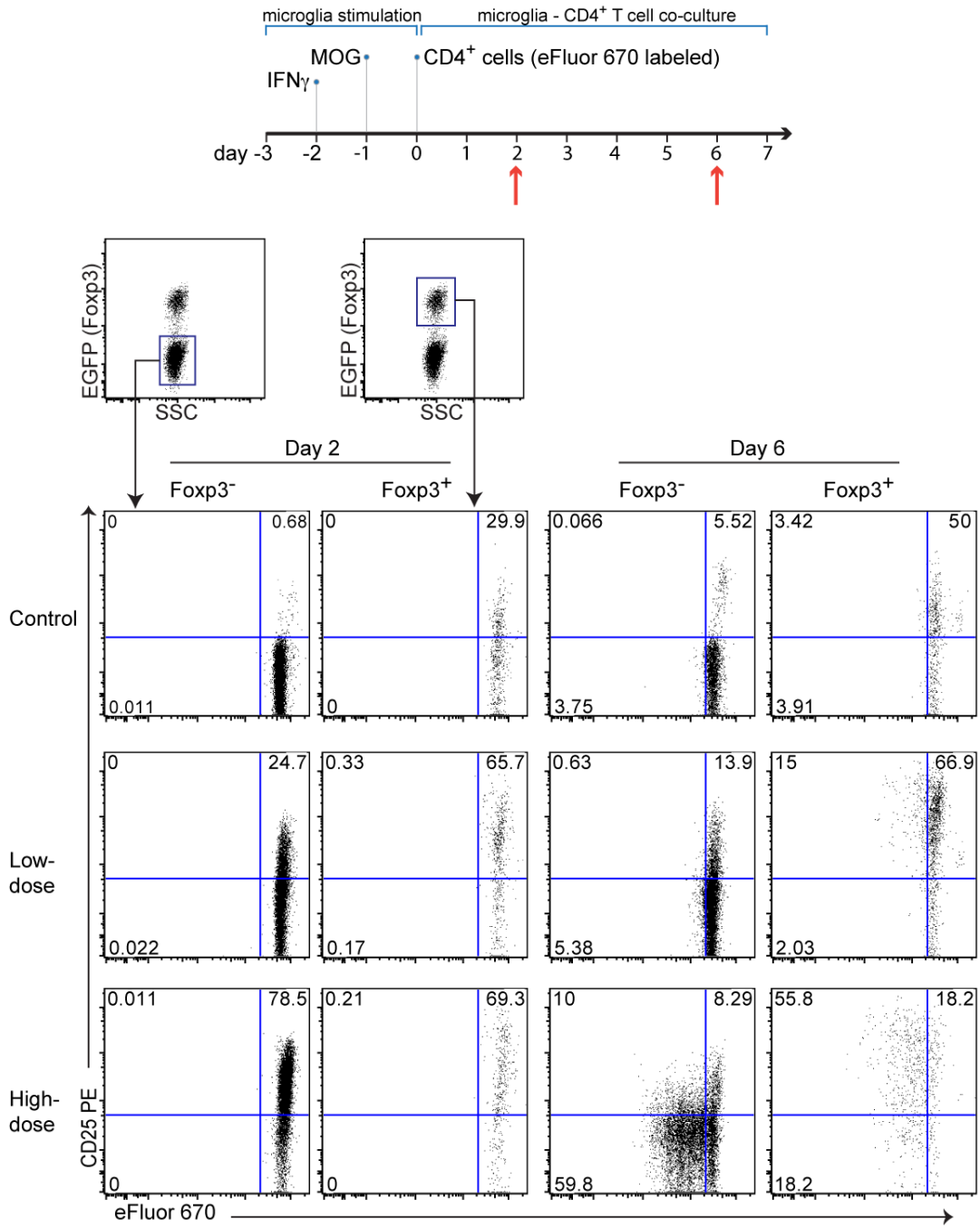

Fig. 3.7 Activated microglia induce cell proliferation in CD4$^+$ T cells.
CD4$^+$ lymphocytes were labeled with 5 µM eFluor® 670 and co-cultured for 2 days (left panels) and 6 days (right panels) with activated and control microglia. For analysis cells were gated on CD11b$^-$ and CD4$^+$. Data are representative of two separate experiments. Red arrows indicate day of analysis.

3.1.5 Microglia-induced Treg are epigenetically stable

Recently, intrinsic Treg stability was shown to rely on epigenetic modification of the *foxp3* locus[126]. Because *foxp3* demethylation is associated with stable Foxp3 protein expression, we analyzed two highly conserved CpG-rich Treg-specific demethylated regions (TSDR) from Treg cells induced by microglia and thymus derived $CD4^+CD25^+$ nTreg as control cells (**Fig. 3.8**). The extent of TSDR demethylation in microglia-induced Treg was comparable to that of thymus derived nTreg (**Fig. 3.8**), indicating a stable Foxp3 expression for microglia-induced Treg cells.

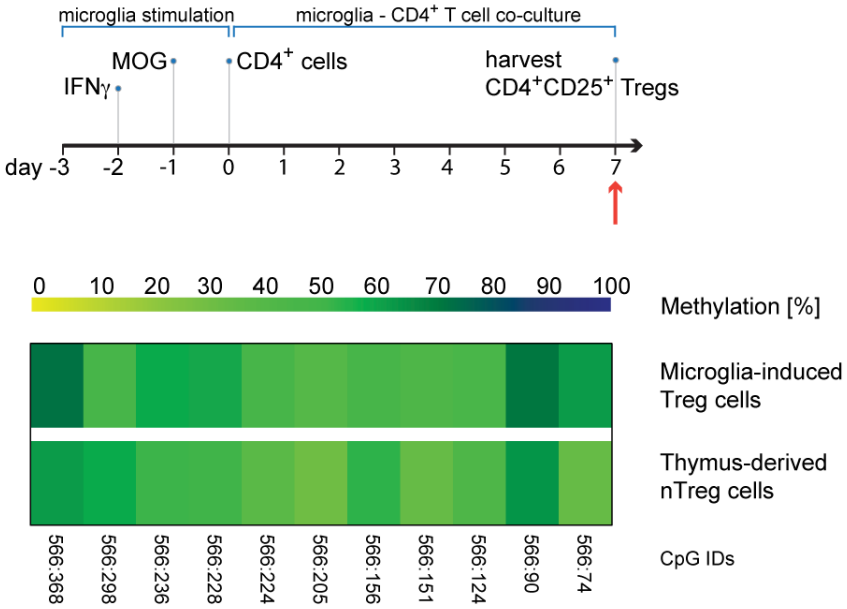

Fig. 3.8 *Foxp3* methylation profile of microglia induced Tregs.
$CD4^+CD25^+$ T cells were harvested 7 days after co-culture with low-dose activated microglia and the DNA-methylation status of two overlapping amplicons within the mouse *foxp3* locus was analyzed using bi-sulfide sequencing (Epiontis GmbH). Single CpG motifs are separated by horizontal lines resulting in boxes representing color-coded methylation rates (yellow = 0%, blue = 100% methylation). Upper panel illustrates microglia-induced Treg cells, lower panel depicts thymus-derived nTreg cells.

3.1.6 Microglia-induced Treg suppress the proliferation of encephalogenic T cells *in vitro*

To investigate the suppressive capacity of microglia-induced Treg cells, we sorted $CD4^{+}CD25^{+}Foxp3^{EGFP+}$ Treg from low-dose activated co-cultures for a MOG-driven suppression assay. Microglia-induced Treg cells efficiently suppressed the proliferation of SNARF-1 labeled responder T cells up to a ratio of 1:50 (**Fig. 3.9 a** upper panel, **b**). As control, we purified $CD4^{+}CD25^{+}Foxp3^{EGFP-}$ from the same cultures and observed no significant inhibitory effect on responder T cell proliferation (**Fig. 3.9 a** lower panel, **b**). These data indicate, that microglia-induced Treg are highly suppressive *in vitro*.

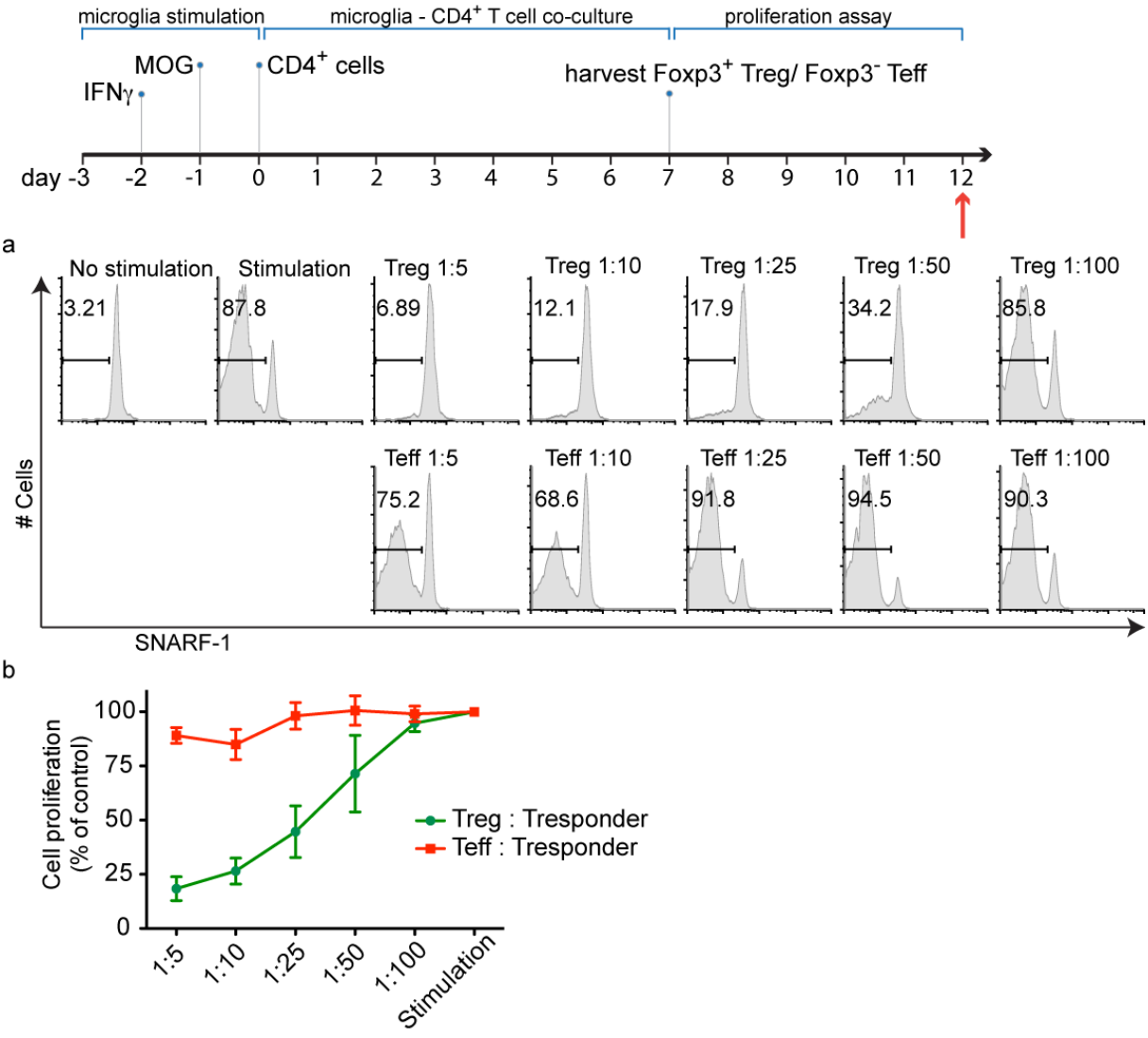

Fig. 3.9 Microglia induce functionally suppressive Treg *in vitro*.
Microglia induced Treg cells were harvested and sorted for $Foxp3^{+}$ Treg and $Foxp3^{-}$ Teffs by flow cytometry. **(a)** shows the proliferation of SNARF-1 labeled responder T cells incubated with irradiated splenocytes and without MOG (no stimulation) or with irradiated splenocytes and MOG in the absence (stimulation) or presence of microglia induced $Foxp3^{+}$ Treg (upper panel) and microglia induced $Foxp3^{-}$ Teffs (lower panel). The ratio of MOG-specific Treg/Teffs : responder T cells is indicated above plots. Numbers above bracketed lines refer to percent of proliferating cells. Data are representative for three separate experiments, summarized in **(b)**, the proliferation index of both groups in percent of control (stimulation).

3.1.7 Microglia-induced Treg reduce EAE severity *in vivo*

Recent studies suggested that the transfer of regulatory T cells isolated from peripheral lymph nodes protects against experimental autoimmune encephalomyelitis (EAE) induction and progression[115]. To examine the effect of microglia-induced Treg cells (miTreg) on the clinical disease course of EAE, we purified MOG-specific $CD25^+$ miTreg and natural Treg (nTreg) from lymph nodes of naive WT animals and adoptively transferred them into C57Bl6/J mice. Control mice received PBS instead. The recipients were immunized with

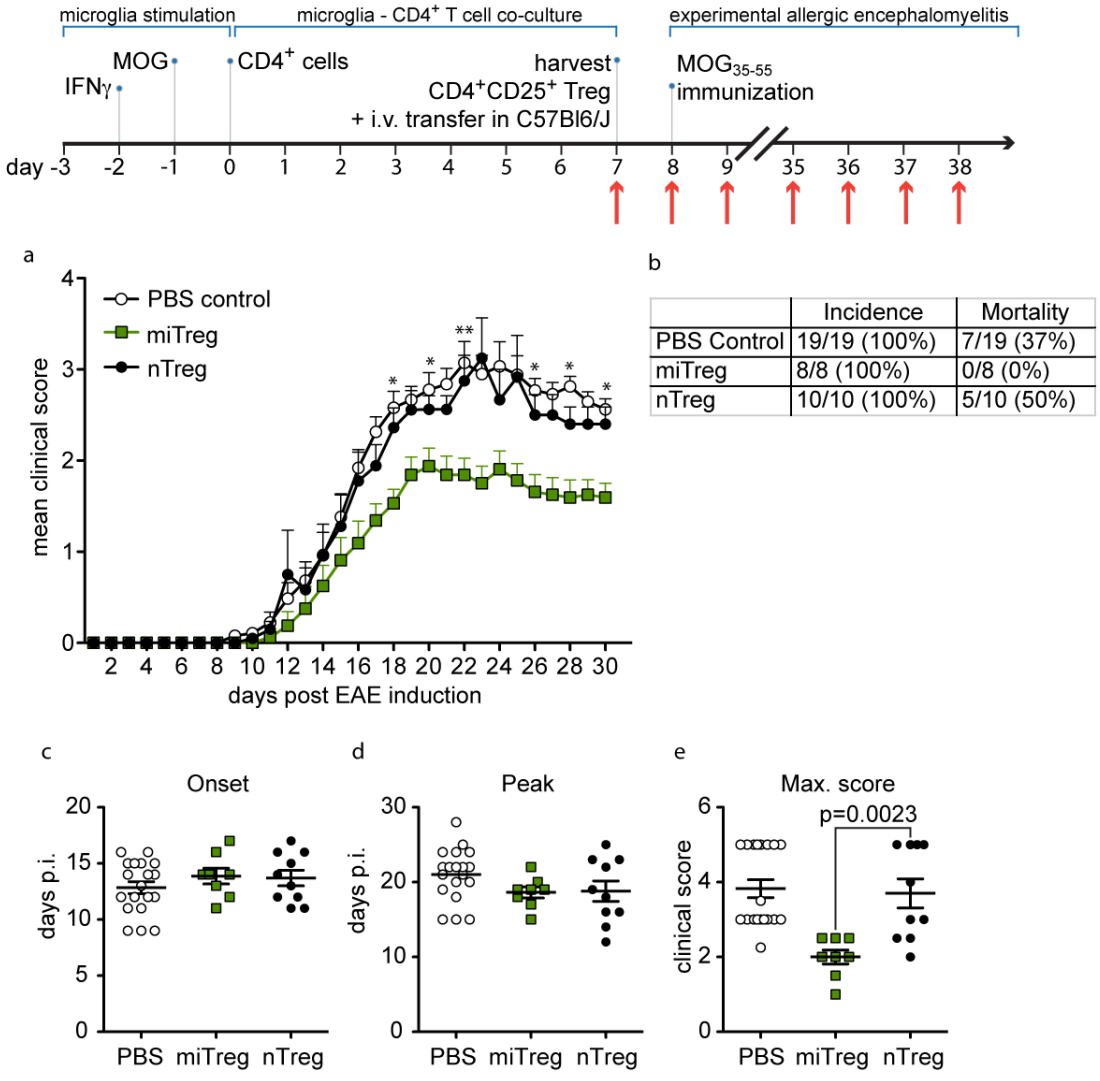

Fig. 3.10 Microglia-induced Treg are functional active *in vivo*.
Microglia-induced Treg (miTreg) or thymus-derived Treg (nTreg) were adoptively transferred in sexmatched C57Bl6/J recipients (5×10^5 cells/mouse). EAE was induced by immunization with MOG_{35-55} peptide in complete Freund´s adjuvant (CFA). The course of EAE disease is shown in **(a)** as mean clinical score (+ SEM) for PBS treated mice (n = 19 open circles), microglia-induced Treg (miTreg) treated mice (n = 8, filled squares) and natural Treg (nTreg) treated mice (n = 10, filled circles) (*p<0.05, **p<0.01, Mann Whitney test comparing miTreg with nTreg). EAE describing statistics are summarized for incidence and mortality **(b)**, mean days of onset post induction (p.i.) **(c)**, mean days of disease peak post induction (p.i.) **(d)** and mean max. clinical score **(e)** (mean ± SEM, Student's *t* test).

MOG_{35-55} in CFA one day after adoptive cell transfer. After immunization of mice, transfer of microglia-induced Treg cells resulted in a constantly reduced clinical disease severity, whereas the transfer of nTreg cells showed no explicit change in clinical scores compared to PBS treated recipients (**Fig. 3.10 a**). In addition, in the group receiving miTreg all mice survived, whereas PBS controls and mice receiving nTreg showed mortality rates of 37% and up to 50%, respectively (**Fig. 3.10 b**). A similar disease onset (between day 12-14) was observed within all groups (**Fig. 3.10 c**). Notably, animals developed classical EAE with 100% incidence (**Fig. 3.10 b**). Together, these results prove that microglia-induced Treg cells are functionally suppressive *in vivo*.

3.2 Results (II) – Characterization of microglial phenotypes

This part is focused on the different microglia phenotypes acquired during activation and illustrates the importance of MHCII and co-stimulatory molecules and the role of IL-10 in modulating microglia phenotype. Furthermore microglia antigen presenting properties are compared to that of professional APCs and alternative ways in microglia activation are studied.

3.2.1 Microglial induction of Treg is strictly MHCII dependent and antigen-specific

To investigate how microglial MHC class II is affected by different doses of IFNγ and antigen, we analyzed microglia cultures 48 h after treatment for MHCII mRNA and molecule

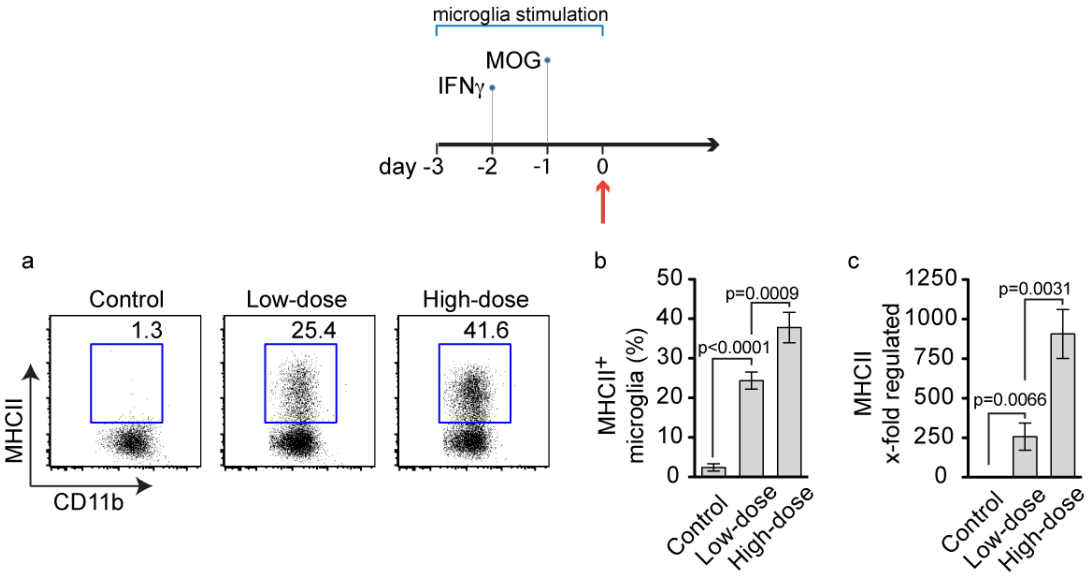

Fig. 3.11 IFNγ induces dose-dependent MHCII up-regulation.
Microglia were analyzed 48 h after IFNγ/MOG stimulation for the expression of MHCII molecules using flow cytometry **(a)**. Numbers above blue gates indicate percent of $MHCII^+$ cells. Data are summarized in **(b)** as percent of $MHCII^+$ microglia gated on $CD11b^+$ cells (n = 4, mean ± SEM, Student's *t* test). **(c)** x-fold increase of MHCII mRNA (n = 3, mean ± SEM, Student's *t* test). Expression levels were normalized to the housekeeping gene *Hprt*.

expression. Dose-dependently, IFNγ/MOG induced a marked increase in MHCII molecule expression on microglia (**Fig. 3.11 a**). While low-dose treated cultures showed 24.38 ± 1.08% MHCII^{+} microglia, high-dose treated cultures reached levels of 37.8 ± 1.92% MHCII^{+} microglial cells (**Fig. 3.11 b**). These data were confirmed by mRNA analyses, showing significant differences in MHCII up-regulation between low-dose (mean 256.7-fold ± 49.35) and high-dose (906-fold ± 89.11) compared to control (**Fig. 3.11 c**).

To confirm that microglial induction of Foxp3^{+} regulatory Treg cells depends on antigen presentation in the context of MHC class II, we used primary microglia derived from MHCII deficient mice (MHCII$^{-/-}$) for *in vitro* co-culture assays. **Fig. 3.12 a** illustrates that in contrast to WT microglia MHCII$^{-/-}$ microglia did not respond to IFNγ stimulation by upregulating MHC

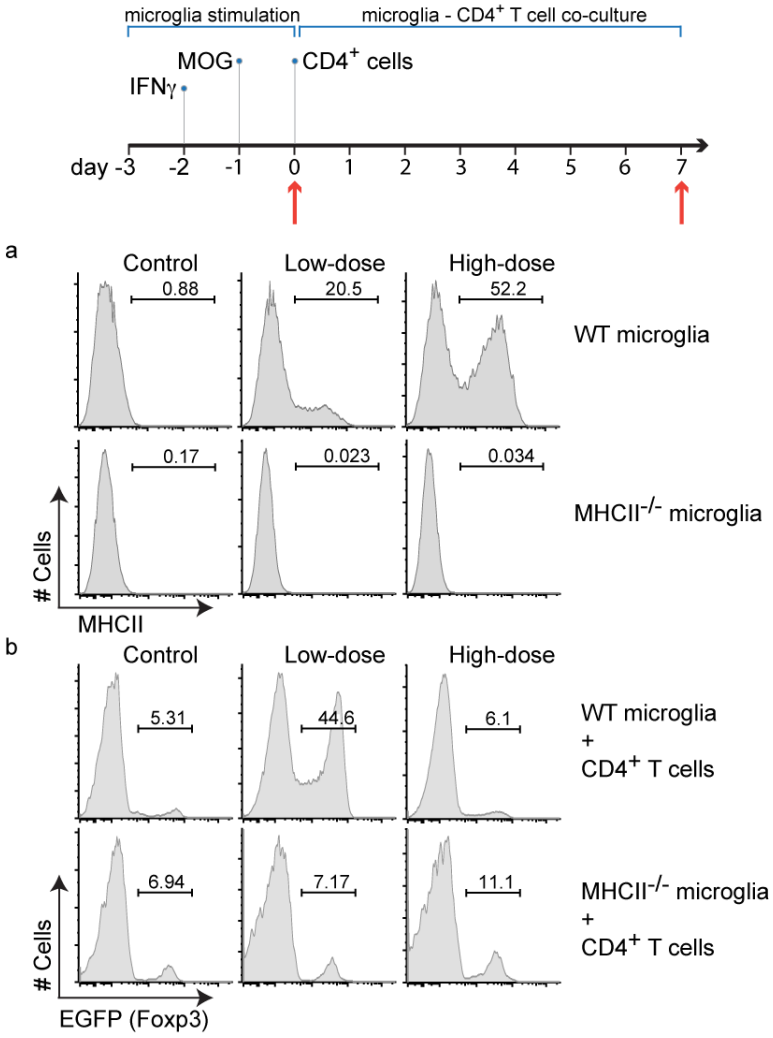

Fig. 3.12 Microglial induction of Foxp3^{+} Treg clearly depends on MHCII expression.
(a) WT and MHCII$^{-/-}$ microglia were pulsed with (low- or high-doses) or without IFNγ/MOG and analyzed for the expression of MHC class II 48 h after stimulation. Numbers above bracketed lines in histograms indicate percent of MHCII^{+} microglia. **(b)** CD4^{+} cells were analyzed for the expression of GFP (Foxp3) after 7 days of co-culture with WT and MHCII$^{-/-}$ microglia. Numbers above bracketed lines in histograms indicate percent Foxp3^{EGFP+} cells. Data are representative of three separate experiments. Red arrows indicate days of analysis.

class II. Experiments with MOG-specific $CD4^+$ T cells revealed that independent of the stimulation level, co-culture with $MHCII^{-/-}$ microglia did not result in induction of $Foxp3^+$ regulatory T cells (7.17% under low-dose treatment), as did low-dose activated WT microglia (44.6%, **Fig. 3.12 b**).

In complementary experiments we analyzed $Foxp3^+$ regulatory T cells from 2D2 mice before and after co-culture with microglia for MOG-transgenic TCR defined by Vβ11 and Vα3.2, to exclude non-antigen specific induction of Treg cells. Analysis of $Foxp3^+$ regulatory T cells, here identified by intracellular Foxp3 staining, revealed 66.3% $V\beta11^+$ Treg cells before and 71% $V\beta11^+$ Treg cells after co-culture (**Fig. 3.13 a**), indicating that microglia induced Treg

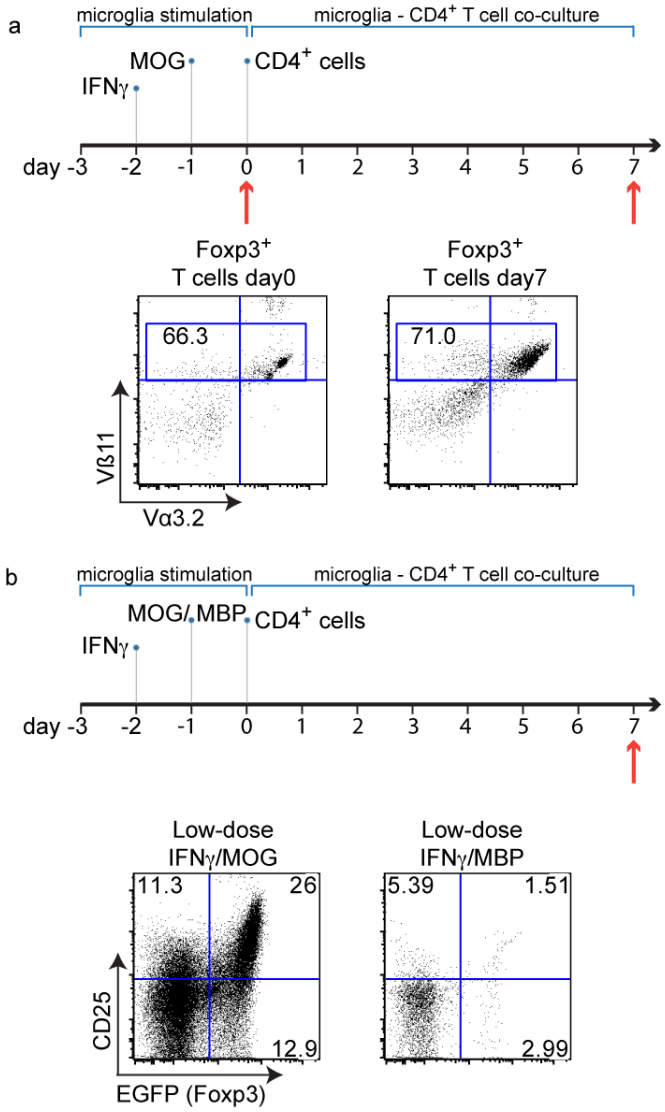

Fig. 3.13 Activated microglia induce antigen-specific $Foxp3^+$ regulatory T cells.
(a) $CD4^+$ T cells were intracellularly stained for $Foxp3^+$ and analyzed for the expression of Vβ11 and Vα3.2 before (left dot plot) and after (right dot plot) co-culture with low-dose activated microglia. Numbers in rectangle gates indicate percent of $V\beta11^+$ cells. **(b)** IFNγ activated microglia were pulsed with MOG (left) or MBP (right) before co-culture. MOG-specific $CD4^+$ cells were analyzed for the expression of GFP (Foxp3) after 7 days. Numbers in left quadrants indicate percent of $Foxp3^+$ cells. Red arrows indicate day of analysis.

display preferentially a MOG-specific TCR. In a second set of experiments, we pulsed IFNγ activated microglia with a different peptide, which is not recognized by the 2D2 transgenic T cell receptor, such as myelin basic protein (MBP). Co-culture of MOG-specific $CD4^+$ T cells and low-dose (IFNγ/MBP) activated microglia did not result in induction of $Foxp3^+$ regulatory T cells (4.5%) in contrast to MOG pulsed cultures (39%, **Fig. 3.13 b**). Together, these results demonstrate that microglia require MHC class II for effective antigen presentation, leading to preferential induction of $Foxp3^+$ regulatory T cells when pulsed with low doses of IFNγ and respective antigen.

3.2.2 Levels of co-stimulatory molecules contribute to tolerogenic phenotype of microglia

CNS microglia up-regulate MHC class II and co-stimulatory molecules (e.g. CD40, CD80, and CD86) as a sign of activation in inflammatory diseases such as MS[127,128]. To further characterize the dose-dependent activation of microglial cells following IFNγ and antigen stimulation, we analyzed the regulation of these co-stimulatory molecules 48 h after treatment. Up-regulation of CD40 and CD86 significantly differed in low-dose and high-dose treated microglial cultures (**Fig. 3.14 a, b**). CD40 expression was 3-fold lower in low-dose cultures when compared to high-dose treated microglia (**Fig. 3.14 a**). Similarly, in low-dose stimulated cultures CD86 expression was 2-fold reduced compared to high-dose treatment (**Fig. 3.14 b**). Interestingly, IFNγ/MOG treatment, independent of its concentration had no

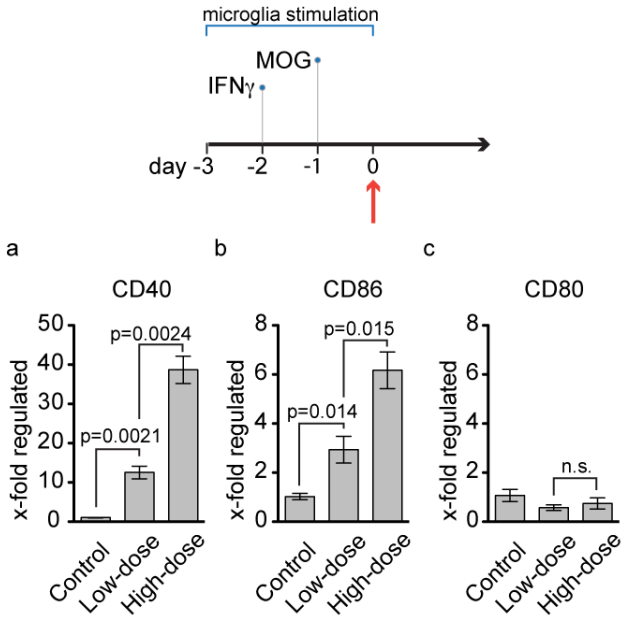

Fig. 3.14 Microglia differentially regulate level of co-stimulatory molecules.
Microglia were analyzed 48 h after IFNγ/MOG stimulation for mRNA expression of **(a)** CD40 (n = 3), **(b)** CD86 (n = 3-4) and **(c)** CD80 (n = 3-4). Expression levels were normalized to the housekeeping gene *Hprt* (mean ± SEM, Student's *t* test).

effect on CD80 expression (**Fig. 3.14 c**). This might indicate, that moderate levels of CD40 and CD86 expression contribute to the tolerogenic phenotype of microglia that preferentially induce regulatory T cells.

3.2.3 CD40-Ligand (CD40L) studies

A recent study reported anti-CD40L treatment (anti-CD154 mAb) to selectively inhibit proliferation of effector T cells, but not $CD4^+CD25^+$ Treg cells *in vitro*, allowing the latter to be expanded[129]. We therefore co-cultured high-dose activated microglia with $CD4^+$ T cells in the presence of anti-CD40L. However, the percentage of $Foxp3^+$ Treg was not affected by the presence of anti-CD40-Ligand (6.4%) compared to high-dose control (5.5%) after 7 days of co-culture (**Fig. 3.15**). In contrast, blocking CD40 using CD154 (CD40-Ligand) resulted in a decrease of $CD25^+$ cell population, indicating, that overall $CD4^+$ T cell activation was affected (**Fig. 3.15**). In addition, overall T cell numbers markedly decreased in co-cultures supplemented with CD40L (data not shown).

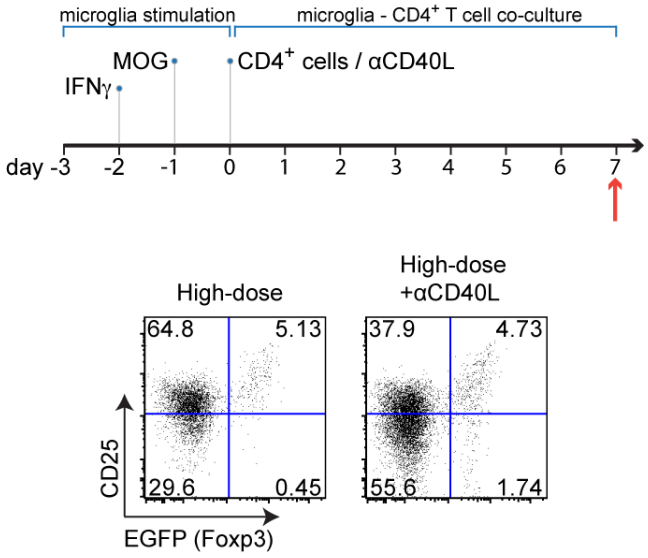

Fig. 3.15 The role of CD40/CD40L interaction in Treg proliferation.
Microglia cultures were stimulated with high-dose IFNγ/MOG (100 U/ml IFNγ/ 10 µg/ml MOG) and co-cultured with $CD4^+$ T cells in the presence of CD40-Ligand antibody (anti-CD154, 40 µg/ml). Dot plots are showing CD25 staining and $Foxp3^{EGFP}$ expression with numbers in quadrants indicate percent of $CD4^+$ gated cells. Data are representative of three independent experiments.

3.2.4 Microglia secrete IL-10 particularly under low-dose conditions

As previously reported interleukin-10 (IL-10) is produced in microglia-T cell co-cultures (see also CBA data from **Fig. 3.4**). In the periphery type II monocytes reveal anti-inflammatory properties characterized by enhanced secretion of IL-10[106]. We therefore analyzed the regulation of *IL-10* gene expression in microglia following IFNγ/MOG stimulation. The

addition of low-dose stimuli resulted in a significant up-regulation of IL-10 compared to control (mean 9.6-fold ±1.8, **Fig. 3.16 a**), whereas high levels of IFNγ/MOG only showed a minor increase in IL-10 mRNA production (mean 2.7-fold ±1.2, **Fig. 3.16 a**). Finding IL-10 mRNA levels exclusively up-regulated under low-dose conditions was consistent with IL-10 protein production analyzed by ELISA (**Fig. 3.16 b**). These findings indicate a potential role for IL-10 in microglia-mediated induction of regulatory T cells and raised the question whether the addition of IL-10 to inflammatory microglia would help to reverse their phenotype.

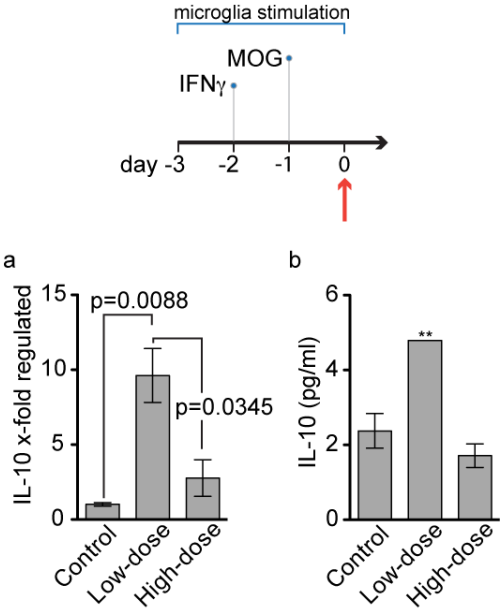

Fig. 3.16 IL-10 production by microglia cells.
a) Microglia were stimulated and analyzed for mRNA expression of IL-10 (n = 3, mean ± SEM, Student's *t* test). Expression levels were normalized to the housekeeping gene *Hprt*. b) Supernatants were collected and analyzed for IL-10 protein levels (ELISA, n = 3, mean ± SEM,**p<0.01, 1-way ANOVA).

3.2.5 IL-10 rescues regulatory T cell response

Because IL-10 is known to affect antigen presentation via modulation of IFNγ-induced MHCII molecule expression[130], we investigated microglial MHCII expression after combined treatment of high-dose IFNγ/MOG and different doses of recombinant IL-10, ranging from 0.2 ng/ml to 20 ng/ml IL-10 (**Fig. 3.17 a**). Increasing levels of IL-10 resulted in a dose-dependent suppression of microglial MHCII expression. Notably, we found the combination of high-dose IFNγ/MOG and 2 ng/ml IL-10 corresponding to low-dose levels of MHCII expression (24.5%, **Fig. 3.17 a**). We therefore used 2 ng/ml IL-10 combined with high-dose IFNγ/MOG treatment for analyzing $CD4^+$ T cell response in microglia-T cell co-cultures. Surprisingly, this combination prevented the differentiation of effector T cells and strongly induced $Foxp3^+$ Treg, in a frequency comparable to that of low-dose IFNγ/MOG stimulation

(32%, **Fig. 3.17 b**). These data demonstrate, that the pre-treatment with IL-10 rescues the regulatory T cell response in co-cultures with inflammatory (high-dose) activated microglia, and indicate a crucial role of IL-10 in inducing a regulatory microglia phenotype.

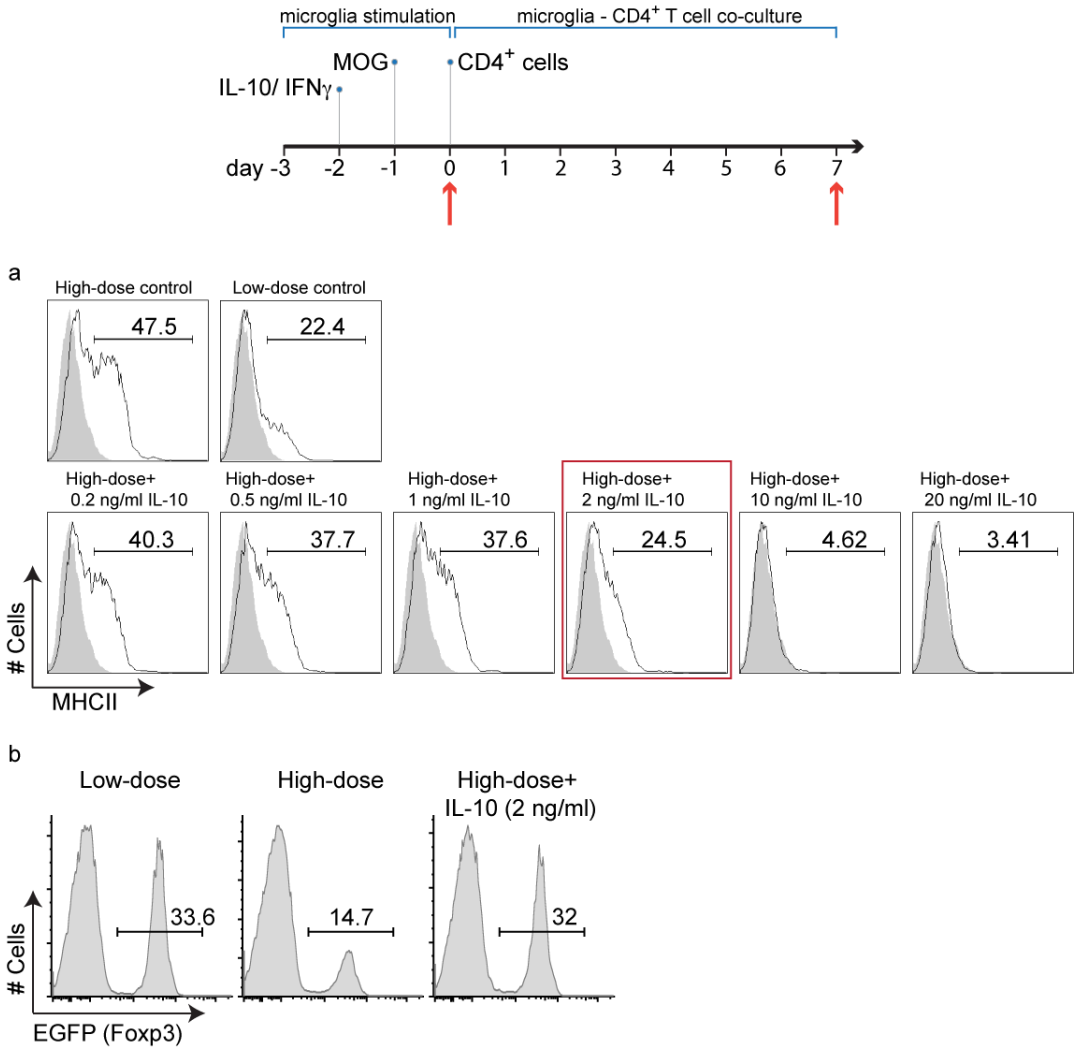

Fig. 3.17 A role for IL-10 in modulating microglia phenotype and CD4$^+$ T cell response.
IFNγ stimulation of microglia was combined with addition of recombinant IL-10 before co-culturing them with CD4$^+$ T cells. **(a)** Histograms show dose response curves of IFNγ induced MHC class II 48 h after stimulation inhibited by increasing levels of anti-inflammatory IL-10. Numbers above bracketed lines indicate percent of MHCII$^+$ cells. Data are representative of four individual experiments. **(b)** Expression analysis of Foxp3$^+$ Treg after 7 days in co-cultures treated with low-dose, high-dose and IL-10 (2 ng/ml) + high-dose IFNγ/MOG. Numbers above bracketed lines indicate percent of Foxp3^{EGFP+} cells. Data are representative of four separate experiments.

3.2.6 IL-10 effects IFNγ-induced microglia activation

To elucidate the effects of IL-10, that render high-dose activated microglia to mediate a regulatory T cell response, we investigated mRNA levels of high-dose IFNγ/MOG activated cells and compared them to high-dose plus IL-10 (2 ng/ml) stimulated microglia. Interestingly,

the regulation of the co-stimulatory molecules CD40, CD80 and CD86 was not affected (**Fig. 3.18**). In turn, exogenous IL-10 resulted in a significant down-regulation of endogenous IL-10 transcription, the chemokine CXCL10, also known as IP-10 and IFNγ inducible NO synthase (iNOS, **Fig. 3.18**).

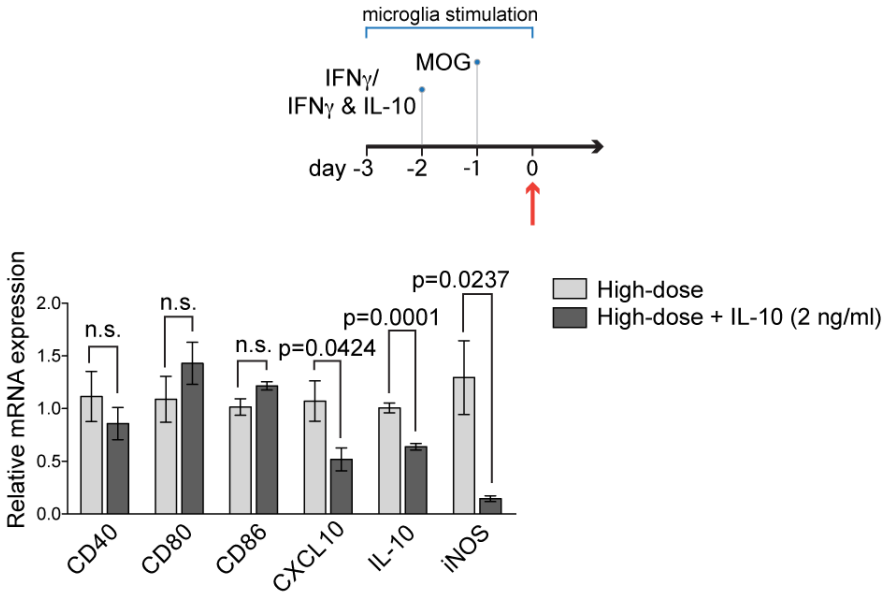

Fig. 3.18 IL-10 modulates microglia activation.
Microglia cultures were analyzed 48 h after high-dose IFNγ/MOG and IL-10 (2 ng/ml) + high-dose IFNγ/MOG treatment for the mRNA expression of co-stimulatory molecules (CD40, CD80, CD86), anti-inflammatory IL-10, IFNγ dependent activation marker (iNOS) and the chemoattractant IP-10/CXCL10 (n = 4-6, mean ± SEM, Student's *t* test). Expression levels were normalized to the housekeeping gene *Hprt*.

3.2.7 Microglial antigen presenting properties compared to professional APCs

IFNγ activated microglia were shown to less efficiently prime naive T cells compared to professional dendritic cells (DC)[73]. Finding a microglia phenotype that specifically induced Foxp3$^+$ regulatory T cells from naive precursors raised the question, how the regulatory efficiency of microglia differs from that of professional APCs. Therefore, we applied the same stimulations and co-culture conditions to bone marrow derived macrophages (BM-M) and dendritic cells (BM-DC) and analyzed MHCII expression after stimulation and the induction of Foxp3$^+$ Treg cells after 7 days. As expected, the control level of MHCII expressing cells was strikingly increased in BM-M (**Fig. 3.19 b**, mean 15.3 ± 0.89%) and BM-DC (**Fig. 3.19 c**, mean 54.7 ± 3.46%) compared to microglia (**Fig. 3.19 a**). IFNγ/MOG stimulation further increased the percentage of MHCII$^+$ cells up to 63.42% in BM-M (**Fig. 3.19 b**) and 86.36% in BM-DC (**Fig. 3.19 c**) cultures, respectively. Surprisingly, we found no significant differences between low-dose and high-dose stimulation conditions in BM-M and BM-DC cell subsets, indicating that microglial MHCII expression is lower but regulated more differentiated.

Low-dose activated BM-DC induced the largest pool of Foxp3$^+$ Treg (49%, **Fig. 3.19 d**) from CD4$^+$ T cells after 7 days of co-culture. Co-culture with low-dose activated BM-M resulted in induction of 34% Foxp3 expressing Treg, comparable to microglial cells (32%, **Fig. 3.19 d**). When stimulated with high-doses of IFNγ/MOG, the frequency of Foxp3$^+$ Treg clearly decreased in all cell subsets. However, in contrast to high-dose activated microglia, BM-M and also BM-DC still induced a distinct frequency of Foxp3$^+$ Treg cells (BM-M 26% and BM-DC 33%, **Fig. 3.19 d**), respectively. These data reflect, that microglial activation is a finely tuned process balancing regulatory and inflammatory CD4$^+$ T cell responses.

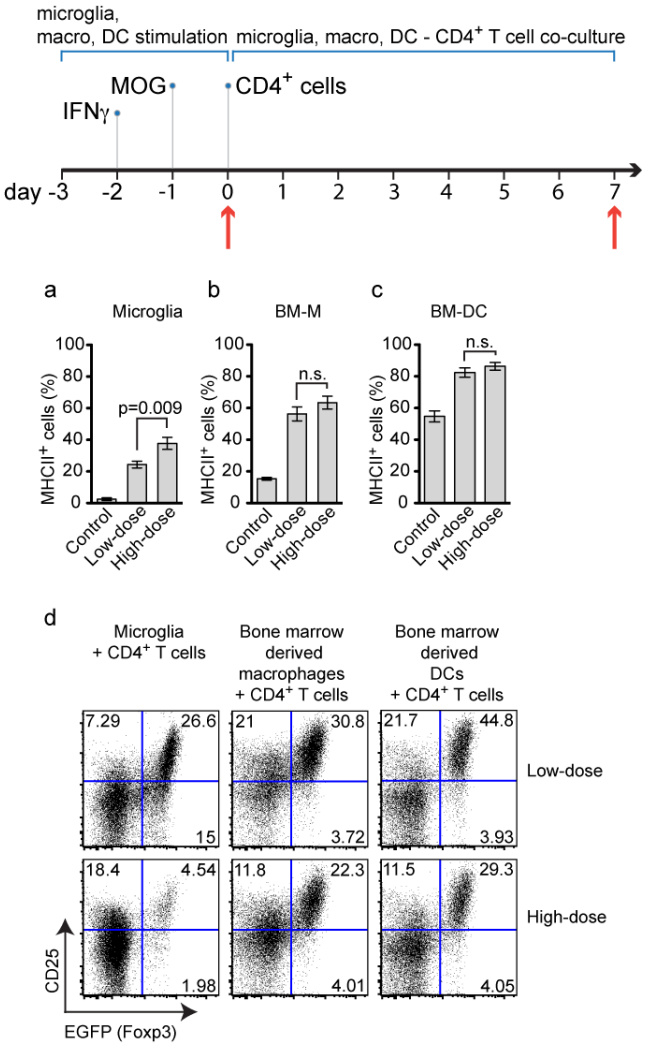

Fig. 3.19 Antigen presentation and CD4$^+$ T cell response of microglia and professional APC.
Microglia, bone marrow derived macrophages (BM-M) and dendritic cells (BM-DC) were generated *in vitro* from C57Bl6/J mice. Microglia **(a)**, BM-M **(b)** and BM-DC **(c)** were stimulated with low- and high-dose IFNγ/MOG and analyzed after 48 h for expression of MHC class II by flow cytometry. Data are presented as mean ± SEM with n = 4-15 using Student's *t* test. **(e)** Flow cytometric analysis of CD25 and Foxp3 expression on CD4$^+$ T cells co-cultured with respective APCs. Data are representative of two separate experiments.

3.2.8 Alternative ways to activate microglia cells

Considering that stimulation with recombinant IFNγ worked well as a danger signal leading to up-regulation of antigen presenting molecules on microglia *in vitro*, we replaced recombinant IFNγ in our systems with Th1 cells or Th1 cell supernatant from WT mice. Therefore, we generated Th1 cells *in vitro*, harvested Th1 cells and supernatants on day 4-5 after preparation, stimulated microglia with a gradient of both and analyzed MHC class II expression after 48 h. Th1 cell titration ranged from 6 x 10^4/ml up to 2 x 10^6/ml, while Th1 cell culture supernatants were diluted stepwise down to 1:32. We observed that supernatants from activated Th1 cells induced MHC class II expression on microglia in a concentration-dependent way (**Fig. 3.20 a**). Notably, cultures treated with the undiluted Th1 cell supernatants showed up to 50% MHCII$^+$ microglia, whereas the 1:32 dilution resulted in only 4.5% MHCII$^+$ cells. MHCII$^+$ microglia frequencies corresponding with low-dose IFNγ/MOG treated cultures were achieved with a 1:4 dilution of Th1 supernatant (24% MHCII$^+$ microglia). In contrast, higher concentrations of Th1 cells on microglia (2 x 10^6/ml and 1 x 10^6/ml) led to rather low MHCII$^+$ frequencies (**Fig. 3.20 b**) and a massive loss of microglial cells was observed in parallel. The stimulation with around 1.25 x 10^5/ml Th1 cells resulted in MHCII$^+$ frequencies similar to that of low-dose recombinant IFNγ treatment (**Fig. 3.20 b**). In complementary experiments we stimulated microglial cells with substances already described or speculated to induce MHC class II on microglia *in vitro*, such as CpG DNA[131], Hsp60, LPS[132,133], poly I:C or necrotic astrocytes. However, with none of these substances microglial MHCII expression could be detected by flow cytometry. These data indicate that antigen presenting capacities of brain resident microglia are particularly regulated by type II interferons (primarily IFNγ).

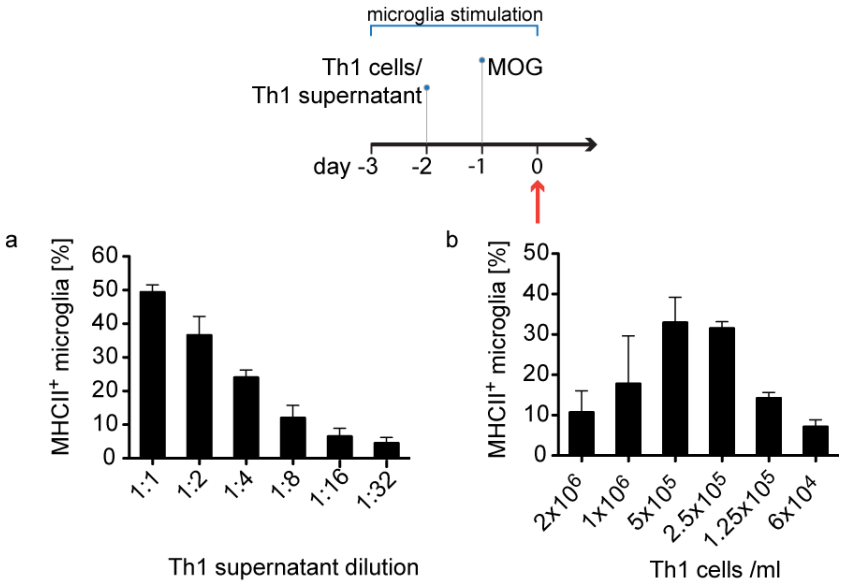

Fig. 3.20 Th1 cells and Th1 cell supernatant induce MHC class II expression on microglia.
Flow cytometry analysis of MHC class II expression on microglia stimulated with a gradient of **(a)** Th1 cell supernatant (n = 3, mean ± SEM) or **(b)** Th1 cells (n = 3, mean ± SEM). Red arrow indicates day of analysis.

3.2.9 CD4$^+$ T cell co-culture with adult microglia

Analogues to co-cultures with postnatal microglia, we applied adult microglia *in vitro* to substantiate our findings. U. K. Hanisch provided the protocol for generating and culturing adult microglia from C57Bl6/J mice. Adult microglia were cultured on a postnatal, astrocytic layer in the presence of L929 cell conditioned media and harvested for CD4$^+$ T cell co-culture assays 7 days later. Adult microglia were stimulated exactly the same way as postnatal microglia with different doses of IFNγ/MOG.

Whereas unstimulated cells (Control) did not express MHCII, IFNγ/MOG stimulation induced MHCII on up to 81.5 ± 1.8% (**Fig. 3.21 a**, high-dose stimulation) of microglia. Low-dose stimulated adult microglia showed only marginal lower MHCII expression (75 ± 2.3%, **Fig. 3.21 a**). Similar to postnatal microglia, adult microglia induced preferentially Foxp3$^+$ regulatory T cells, when stimulated with tolerogenic doses of IFNγ/MOG (44%, **Fig. 3.21 b**). In contrast, co-cultures with high-dose activated adult microglia showed lower levels of Foxp3$^+$ Treg induction, but still induced a distinct frequency (22%, **Fig. 3.21 b**) compared to postanatal microglia (**Fig. 3.1 a, b**). As a major difference, high-dose activated adult microglia did not evoke distinct CD25 expression on Foxp3$^-$ T cells. It remains unclear, whether this reflects incomplete activation of CD4$^+$ T cells.

Together this data indicate, that adult microglia strongly respond to IFNγ/MOG with up-regulating MHCII and that adult microglia are able, similar to postnatal microglia, to induce Foxp3$^+$ regulatory T cells under low-dose conditions.

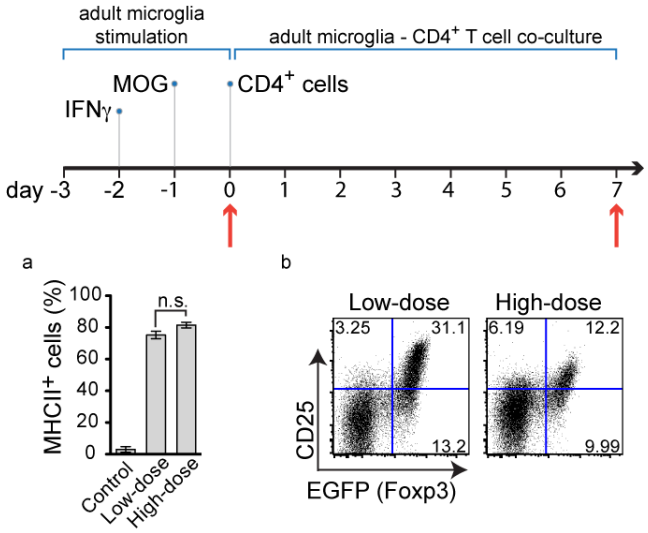

Fig. 3.21 Adult microglia induced T cell responses *in vitro*.

(a) Adult microglia were analyzed 48 h after IFNγ/MOG stimulation for expression of MHC class II molecules by flow cytometry (n = 5, mean ± SEM, Student's *t* test). **(b)** Flow cytometric analysis of CD25 and Foxp3 (GFP) expressing cells after 7 days of co-culture with adult microglia. Data are representative of two individual experiments.

3.3 Results (III) – Potential *in vivo* implications of MHCII expression within the CNS

This part aims at the influence of CNS MHCII expression on $CD4^+$ T cell responses in a mouse model of brain injury. The entorhinal cortex lesion (ECL) model is widely used to study microglial activation and leukocyte infiltration after CNS injury. The model involves the stereotactical lesion of the perforant path, a myelinated fiber tract connecting the entorhinal cortex with the hippocampus, leading to anterograde degeneration of the hippocampus[54]. After ECL, myelin-specific T cells expand in draining lymph nodes, invade regions of degeneration but do not elicit autoimmune encephalomyelitis-like white matter damage. Inflammation in regions of degeneration, as it occurs in MS, seems to be tightly regulated and eventually kept under control after brain injury, implying active maintenance of immune tolerance[54]. We therefore used ECL to investigate whether antigen presentation properties of brain resident cells alter the $CD4^+$ T cell response to CNS tissue damage.

3.3.1 Enhanced CNS infiltration of $CD4^+$ T cells and $Foxp3^+$ Treg after ECL is prevented in $MHCII^{-/-}$ mice

In preliminary experiments we demonstrated that among CNS infiltrating T lymphocytes detected after entorhinal cortex lesion (ECL), a model of sterile CNS injury, $CD4^+$ T cells and especially $CD4^+Foxp3^+$ Treg cells significantly increased in number and frequency 14 days after the insult at the site of lesion. At the same time increased numbers of MHCII expressing $Iba1^+$ cells were detected in the lesion site and in direct contact to $Foxp3^+$ Treg cells (Stubbe, personal communication). We therefore asked whether CNS expression of major histocompatibility complex class II (MHCII) is required for accumulation of regulatory T cells in a model of non-inflammatory CNS lesion (ECL). To address this issue we operated MHC class II deficient mice ($MHCII^{-/-}$), which received 2 x 10^7 $CD4^+$ T cells from $Foxp3^{EGFP}$ reporter mice 1 day before ECL to reconstitute the $CD4^+$ T cell compartment, and analyzed $CD4^+$ and $CD4^+Foxp3^+$ T cells according to the gating strategy illustrated in **Fig. 3.22**. In contrast to the WT situation, we observed no significant CNS-infiltration of $CD4^+$ lymphocytes in ipsilateral compared to contralateral hemispheres in $MHCII^{-/-}$ mice (**Fig. 3.23 a**). In line with that, ipsilateral numbers and frequencies of infiltrating $CD4^+Foxp3^+$ Treg cells were drastically reduced in $MHCII^{-/-}$ mice (**Fig. 3.23 b** and **c**) and did not differ between ipsi- and contralateral hemispheres. This indicates that the infiltration of $CD4^+$ lymphocytes and the accumulation of $Foxp3^+$ Treg cells at the site of CNS lesion is an active response of the adaptive immune system requiring antigen presentation via MHC class II.

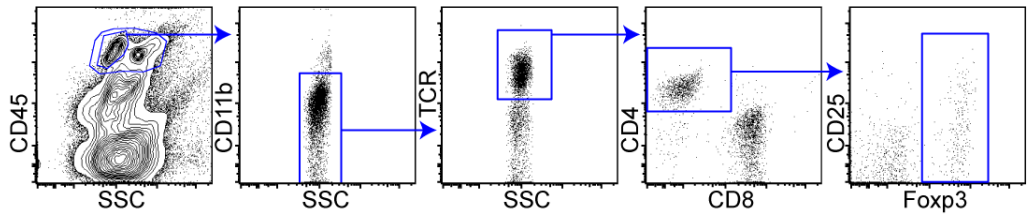

Fig. 3.22 Gating strategy for flow cytometry analysis of CNS infiltrating leukocytes.

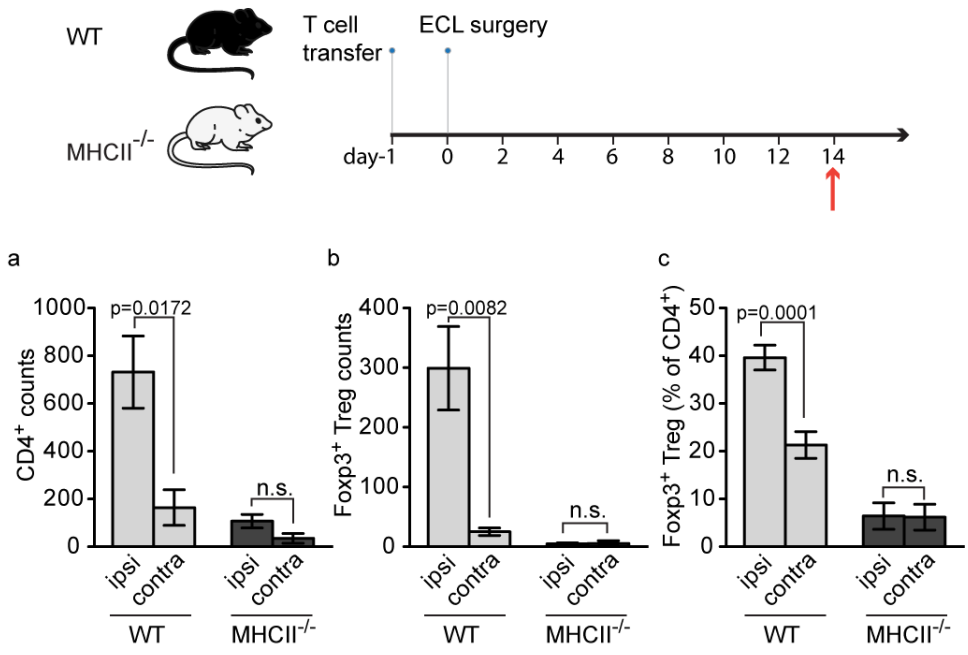

Fig. 3.23 Foxp3$^+$ Treg cells infiltrate the lesioned CNS of WT but not MHCII-deficient mice.
Absolute numbers of infiltrating CD4$^+$ (a) and CD4$^+$Foxp3$^+$ Treg cells (b) 14 days after ECL in WT vs. MHCII$^{-/-}$ mice analyzed by flow cytometry (n = 7-9, mean ± SEM, Student's paired t test). (c) Flow cytometry of Foxp3^{EGFP} frequency in CD4$^+$ T cells, comparing ipsilateral and contralateral hemispheres of WT and MHCII$^{-/-}$ mice (n = 7-9, mean ± SEM, Student's paired t test).

3.3.2 Diminished Foxp3$^+$ Treg cell recruitment after ECL in mice lacking MHCII specifically in the CNS

To study the role of MHC class II restricted antigen presentation by CNS resident cells and its effect on CD4$^+$Foxp3$^+$ Treg cell recruitment and accumulation, we generated bone marrow chimeric mice (**Fig. 3.24**) deficient of MHCII exclusively in the periphery (MHCII$^{-/-}$ → WT) or in the CNS (WT → MHCII$^{-/-}$). As MHCII WT control we used Foxp3^{EGFP} mice reconstituted with Foxp3^{EGFP} bone marrow referred to as WT → WT chimeric mice (**Fig. 3.24**).

Following engraftment, blood phenotyping revealed that B220$^+$ B cells of WT mice reconstituted with MHCII$^{-/-}$ bone marrow (MHCII$^{-/-}$ → WT) expressed almost no MHC class II (1.2%), whereas WT → MHCII$^{-/-}$ chimeras expressed with 96.1% MHCII already wild-type

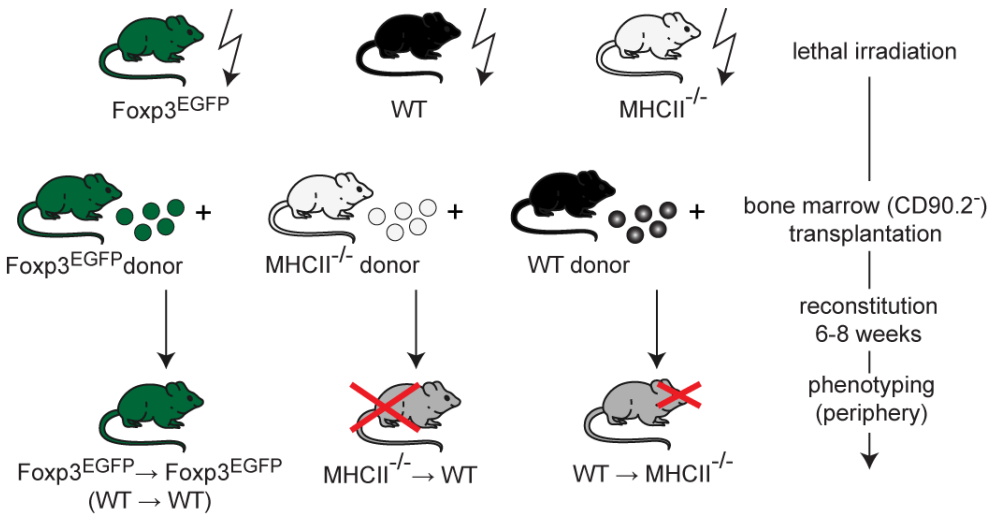

Fig. 3.24 Experimental setup for generating bone marrow chimeras.
Following lethal irradiation mice were transplanted with respective bone marrow depleted of $CD90.2^+$ (Thy1.2) cells and allowed to reconstitute for 6-8 weeks before they were subjected to blood phenotyping.

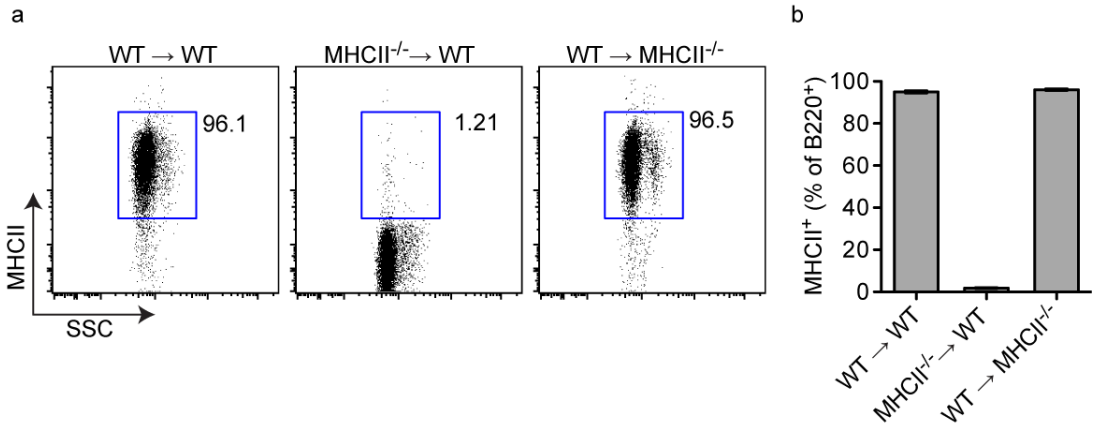

Fig. 3.25 Blood typing of MHCII bone marrow chimeras.
Lymphocytes isolated 6 weeks after reconstitution from the blood of chimeric mice were subjected to flow cytometry **(a)** Dot plots showing MHCII staining of $B220^+$ cells of all three groups. Numbers beside quadrant gates indicate CD45R/$B220^+$MHCII$^+$ cells. Dot plots are representatives of 8-18 mice per group of chimeras summarized in **(b)** as MHCII$^+$ expressing cells in % of $B220^+$ cells (mean ± SEM).

WT → WT levels (96.5%, **Fig. 3.25 a**). Typing results are summarized in **Fig. 3.25 b** and support that WT → MHCII$^{-/-}$ chimeras harbor a normal peripheral APC compartment (MHCII expression on B cells, macrophages and DCs), but lack MHCII expression on CNS resident cells.

While WT → WT and WT → MHCII$^{-/-}$ chimeras recovered well after reconstitution, MHCII$^{-/-}$ → WT chimeric mice developed symptoms of ulcerative colitis (e.g. diarrhea and prostrated posture) 7-8 weeks after reconstitution and died within a few days after the first symptoms.

Since Marguerat *et al*. already described these findings as a murine radiation-induced colitis model[134], we tried to overcome immunopathology by applying different strategies. We repeated the generation of MHCII$^{-/-}$ → WT chimeras with prolonged antibiotic treatment (Baytril®) on one hand or administered regulatory T cells from congenic (Thy1.1) mice i.v. on the day of reconstitution to enlarge the pool of protective T lymphocytes on the other hand. However, none of these strategies worked out and only WT → WT and WT → MHCII$^{-/-}$ chimeric mice were subjected to entorhinal cortex lesion (ECL) and analyzed 14 days later according to **Fig. 29**. Notably, both groups received 2 x 10^7 CD4$^+$ T cells from Foxp3^{EGFP} reporter mice one day before they were subjected to ECL surgery.

We analyzed the composition of brain infiltrating leukocytes, cervical lymph nodes (CLN) and blood with special interest in CD4 and Foxp3 expressing lymphocytes. We compared infiltrated numbers of CD45^{high} cells ipsilateral (**Fig. 3.27 a**) from WT → WT (mean 5221 ± 1182 cells) and WT → MHCII$^{-/-}$ mice (mean 4802 ± 540 cells) and found no significant difference in CD45^{high} cell number, indicating that local MHCII expression is not crucial for overall leukocyte infiltration. In contrast, the number of CD4$^+$ cells were significantly decreased in WT → MHCII$^{-/-}$ mice (mean 339 ± 41 cells) compared to WT → WT animals (mean 619 ± 134 cells) suggesting that mice lacking MHCII in the CNS are no longer able to selectively recruit CD4$^+$ T cells to the lesion site (**Fig. 3.27 b**). In line with that, we also found the number of Foxp3 expressing Treg cells (**Fig. 3.27 c**) to be diminished in WT → MHCII$^{-/-}$

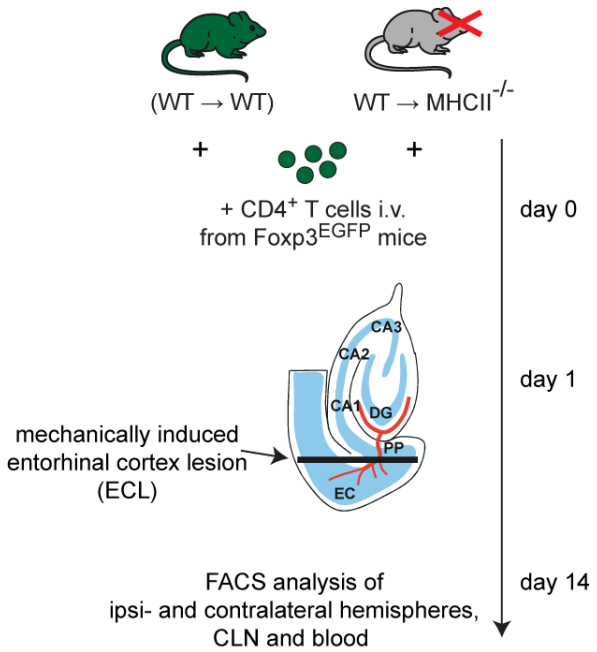

Fig. 3.26 Experimental setup for inducing CNS injury (ECL) in MHCII chimeric mice.
EC = Entorhinal Cortex, DG = Dendate Gyrus, PP = Perforant Path, CA1-3 = Cornu Ammonis, CLN = Cervical Lymph Nodes.

mice (mean 106 ± 19 cells) versus WT → WT animals (mean 169 ± 43 cells). Related to overall infiltrated leukocytes, we observed that significant less Foxp3$^+$ Treg cells per CD45^{high} leukocyte were recruited to the ipsilateral hemispheres of WT → MHCII$^{-/-}$ versus WT → WT chimeras (**Fig. 3.27 d**), implying an potentially less regulatory CNS infiltrate in mice lacking MHCII specifically in the brain. In addition, we compared Foxp3$^+$ Treg frequencies of the brain with that of CLN and blood but found no longer a specific increase of Treg at the lesion site in WT → MHCII$^{-/-}$ (**Fig. 3.27 e**). We therefore speculate, that MHCII expression in the CNS is required to selectively recruit more CD4$^+$ cells, containing a large proportion of Foxp3$^+$ Treg cells, to the sites of CNS injury. Thus, MHCII expressed on brain resident cells renders the profile of the infiltrating leukocyte population more regulatory in the model of non-inflammatory ECL.

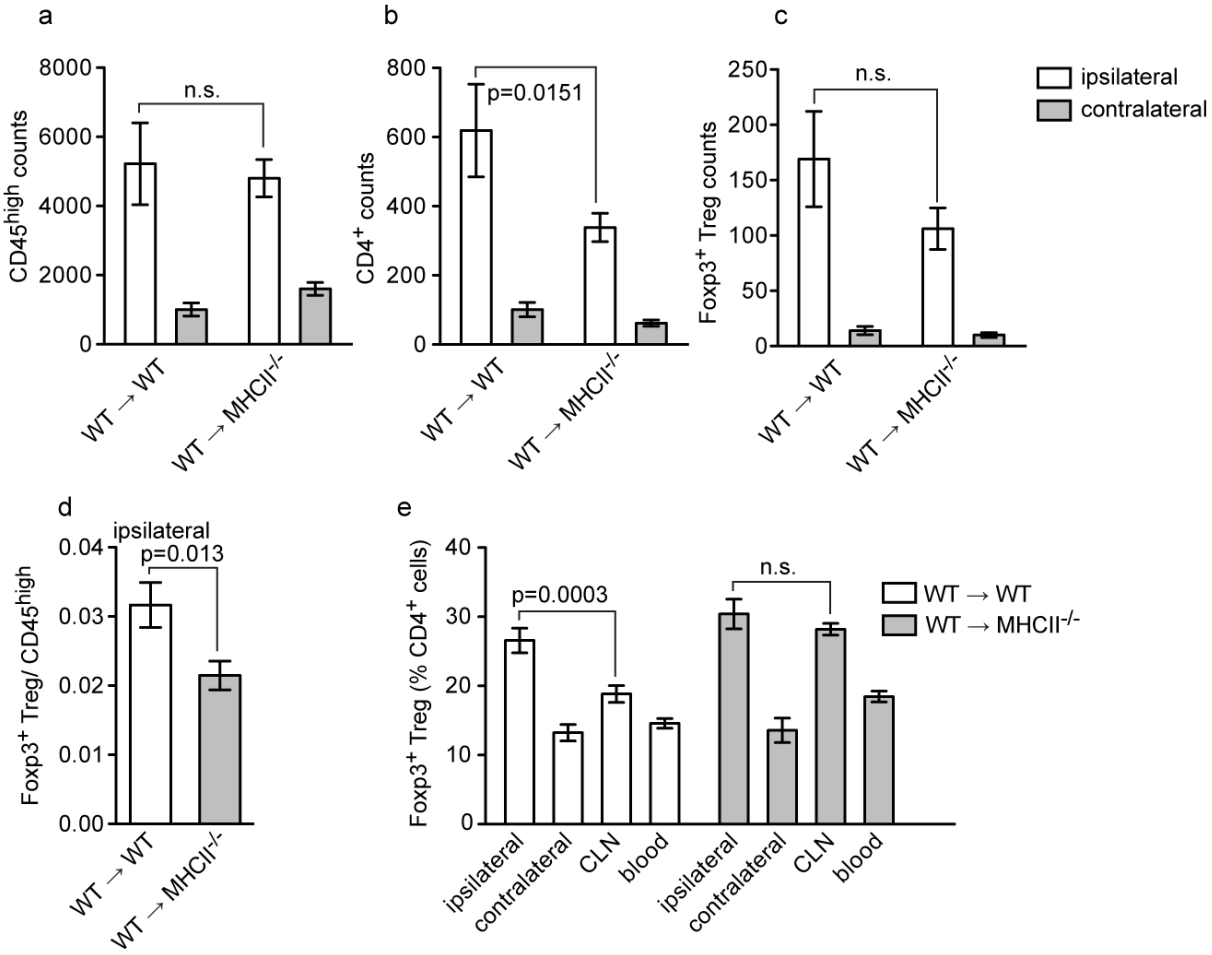

Fig. 3.27 Analysis of CNS infiltrating leukocytes in MHC class II chimeric mice.
CNS infiltrating leukocytes as well as CLN and blood cells from WT → WT and WT → MHCII$^{-/-}$ mice were analyzed by flow cytometry showing absolute numbers of **(a)** CD45^{high}, **(b)** CD4$^+$ and **(c)** Foxp3$^+$ Treg cells and **(d)** the ratio of Foxp3$^+$ Treg per infiltrating CD45^{high} leukocyte (n = 8-18, mean ± SEM, Student's *t* test). **(e)** shows the ratio of Foxp3$^+$ Treg in % of CD4$^+$ T cells comparing CNS infiltrating cells to those from CLN and blood (n = 8-18, mean ± SEM, Student's paired *t* test).

4 Discussion

The trafficking of immune cells into the central nervous system is tightly controlled to maintain immune homeostasis and optimal brain function[135]. While leukocyte extravasation into the CNS is a key event in the pathogenesis of acute or chronic inflammatory neurological diseases[136], CNS immune surveillance is central to protect against cerebral infection[135]. Immune cells of peripheral origin, such as T cells, macrophages and dendritic cells, but also brain resident microglia carry out routine CNS immune surveillance. Microglia, the major immune competent cells of the brain, continuously survey their environment for any stranger or danger signals indicating neuronal disturbances. Lacking cell surface expression of MHC class II and co-stimulatory molecules under steady state conditions, microglia are often referred to as non-professional antigen presenting cells. The regulation of microglia antigen presenting capacity and its effect on controlling T cell responses was the subject of investigation in this thesis. The main findings therein are, that

a) depending on their activation level, microglia mediate the differentiation and proliferation of Foxp3$^+$ Treg or Foxp3$^-$ effector T cells,

b) microglia-induced Treg cells are stable and functionally active *in vitro* and *in vivo*,

c) microglia-mediated Treg induction is MHCII and antigen-dependent,

d) microglia phenotypes control CD4$^+$ T cell responses by regulating levels of co-stimulatory molecule expression and IL-10 secretion.

4.1 The brain – From the T cell's point of view

This study describes the role of microglia phenotypes in modulating CD4$^+$ T cell responses based on an *in vitro* co-culture assay. However *in vivo*, CD4$^+$ T cell extravasation into the CNS and microglial activation are the major prerequisites for microglia to serve as antigen presenting cells and modulate CD4$^+$ T cell responses. Since the resulting immune response of microglia – T cell interaction in the *in vivo* situation also depends on the nature of CNS infiltrating CD4$^+$ T cells, the following section briefly summarizes how T cells gain entry into the CNS, their routes of entry and in which state they are behind the barriers distinguishing between routine immune surveillance and CNS pathology.

There is rising evidence for continuous leukocyte trafficking into the healthy CNS across the endothelial BBB via the perivascular space or across the blood-cerebrospinal fluid barrier (BCSFB) formed by epithelial cells of the choroid plexus[12,13,137]. Studies in different models proof that in the absence of inflammation and independent from antigen-specificity, highly activated but not resting T cells can migrate across the vascular walls of the BBB[6,138–140].

Conflicting findings come from the group of Joan M. Goverman showing that in $Rag^{-/-}$ MBP TCR transgenic mice the vast majority of T cells in the healthy CNS exhibit a naive phenotype[141]. They speculated that these CNS specific T cells undergo tolerance induction *in situ* directly in the CNS and therefore do not trigger autoimmunity[141]. The functional state and lineage commitment *versus* plasticity of infiltrating $CD4^+$ T cells is of particular importance for discussing *in vivo* implications of microglia-mediated induction of $Foxp3^+$ Treg cells (see section **4.3.1**).

In the current model of immune surveillance, CNS patrolling leukocytes are thought to screen antigen-presenting cells in the perivascular space. Besides microglial cells, meningeal and perivascular macrophages, dendritic cells and choroid plexus macrophages are strategically localized adjacent to the brain barriers and capable of antigen presentation via MHC molecules[13], implying that CNS patrolling $CD4^+$ T cells can encounter microglia not only in the deep brain parenchyma but also in the perivascular space. T cells do not seem to further migrate across the glia limitants into the parenchyma in the absence of antigen-triggered re-activation[140], indicating that immunosurveillance is confined to perivascular CNS compartments[12]. Using intravital microscopy during passive EAE, Bartholomäus and colleagues showed that in the absence of their cognate antigen, OVA-specific T cells crossed the vessels but formed only transient contacts with perivascular/meningeal APCs without up-regulation of activation markers or inflammatory cytokines[140]. In contrast, transferred MBP-specific T cells formed diverse and often long-lasting contacts, became re-activated and migrated deeply into the CNS parenchyma. These observations suggest that continuous antigen-specific re-activation is necessary for encephalitogenic T cells to enter the CNS tissue in the absence of inflammation.

In CNS pathology, T cells become re-activated in perivascular or subarachnoid spaces and will trigger an inflammatory response that leads to up-regulation of additional traffic signals on BBB and BCSFB[12]. Recruitment of more and more immune cells together with cytokine and protease secretion will lead to degradation of the glia limitants. As soon as the blood-brain barrier is compromised a large number of inflammatory cells gain entry to the parenchyma and initiate autoimmune CNS inflammation. In contrast to EAE, which models severe CNS inflammation, the model of entorhinal cortex lesion (ECL) reflects a steril, non-inflammatory brain lesion leading to axonal degeneration[54]. Such lesions lead to activation of endothelial cells resulting in a loss of tight junction integrity and formation of transendothelial cell channels[142,143]. In addition to the passive leukocyte entry due to BBB breakage, myelin-specific T cells expand in draining lymph nodes, invade injured regions and mediate secondary degeneration[58,60]. However, brain injury does not lead to destructive autoimmunity implying that mechanisms exist that control T-cell dependent immune responses after CNS trauma[119].

In sum, T cells can actively migrate into the healthy CNS during routine immune surveillance, passively enter the CNS after BBB disruption or are recruited in the context of CNS inflammation. Continuous antigen-specific re-activation of T cells is a critical step during the initial T cell entry in the absence of inflammation as well as for their migration deep into the parenchyma. This is where microglia enter the stage.

4.2 Infiltrating T cells – From the microglial side

Professional APC, that constitutively express MHCII, are missing in the healthy CNS. However brain resident microglia have the potential to migrate to regions of tissue damage, undergo proliferation, increase phagocytic activity, secrete cytokines and chemokines and up-regulate molecules for effective antigen presentation[28]. Assuming that microglial cells do not actively leave the injured CNS to present antigen in draining lymph nodes[144], the microglia-T cell interaction takes place directly behind the brain barriers and in the parenchyma.

We hypothesized that microglia can do both, act as inducer or amplifier of pro-inflammatory $CD4^+$ responses as well as inducer of regulatory mechanisms. This hypothesis was formulated based on previous findings from looking at either the T cell or the APC side. Firstly, in parallel to microglial activation, entorhinal cortex lesion, which models mild inflammatory events, results in infiltration of self-antigen specific T cells that do not initiate MS-like destructive immunity. Quite the contrary is observed; when subjecting lesioned mice to EAE, these animals show reduced disease severity, suggesting long-term tolerogenic effects[119]. Moreover, microglia properties for limiting CNS inflammation or regulating immune responses have been discussed[145]. Secondly, experiments by our group could demonstrated that 14 and 30 days after ECL, $Foxp3^+$ regulatory T cells accumulate in the brain with increased frequencies compared to peripheral organs (Stubbe, unpublished). Immunohistology of ECL brains revealed the presence of MHC class II expressing $Iba1^+$ microglia in contact to $Foxp3^+$ regulatory T cells in the CNS parenchyma. Thirdly, the peripheral induction of $Foxp3^+$ Treg by professional, peripheral APC, such as "subimmunogenic" primed dendritic cells[105] or type II monocytes[106], have been described to specifically rely on their activation-dependent phenotype. To test whether microglia, the immune sentinels of the brain, can mediate conversion of naive $CD4^+$ T cells into regulatory, $Foxp3^+$ expressing T cells, a microglia – T cell co-culture was established.

4.3 Microglia and T cells in co-culture

Microglia are non-professional APC that lack MHC class II molecules in their resting state, but constitutively express the IFNγ receptor (IFNγR). IFNγ is the most prominent inducer and amplifier of cytotoxic, phagocytic, and antigen-presenting features of microglia[146]. Aside from

infiltrates of IFNγ-producing T cells in MS disease or EAE, increased IFNγ expression in the brain has been reported following stroke[112,147,148] and other neuropathological conditions such as nerve injury[149]. T cells ($CD4^+$ and $CD8^+$ phenotype) and natural killer (NK) cells are the main sources of IFNγ, although neurons and microglia themselves have been shown to contribute to CNS IFNγ levels[150,151]. IFNγ signals through binding to the IFNγR, initiating the classical JAK-STAT pathway and leading to transcription of over 200 interferon responsive genes[152,153]. In microglia, IFNγ induces or regulates MHC class I and II molecules[125], co-stimulatory molecules (CD80/86), leukocyte function-associated molecule 1 (LFA-1), LPS receptor (CD14), Fc and complement receptors, changes in the proteasome composition, as well as release of cytokines (TNFα, IL-1, IL-6), complement (C1q, C2, C3, C4), chemokines and NO[146]. Because IFNγ was proven particularly effective as an inducer of MHC molecules[154], recombinant IFNγ was used in this study to induce molecules needed for efficient antigen presentation on microglia. In combination with the brain specific MOG antigen, we were able to analyze TCR-driven differentiation of purified MOG-specific $CD4^+$ T cells mediated by microglial cells. An experimental set up was established, that encompassed IFNγ stimulation for 24 h following MOG-priming for another 24 h before purified $CD4^+$ T cells were applied on top of the microglial monolayer. This timeline allows activation-dependent expression of microglial surface molecules as well as cytokine and chemokine synthesis before T cell engagement.

For the first time this study proves that brain-derived microglia have the ability to induce $Foxp3^+$ regulatory T cells *in vitro* (**Fig. 3.1 a**). $Foxp3^+$ Treg induction was shown to be antigen-specific and MHCII-dependent and, together with the negative correlation between dosage of stimuli/antigen and the $Foxp3^+$ frequency, prompted us to further study microglial plasticity on the one hand and microglia-mediated T cell differentiation on the other hand. The underlying hypothesis was that the high number of $Foxp3^+$ Treg under low-dose conditions is a result of a distinct regulatory microglia phenotype in combination with low levels of antigen presented by these cells.

4.3.1 The T cell side of co-culture

The differential effect of microglia-activation on $CD4^+$ T cell dynamics, their cytokine pattern and the suppressive function of microglia-induced $Foxp3^+$ were studied within the first part of this thesis.

Low-dose treated microglia revealed a very reproducible Treg kinetic with the first increase in $Foxp3^+$ cells showing up 3 days after the beginning of co-culture (**Fig. 3.3**). A similar lag period in single cell Foxp3 protein expression was reported for TGF-β-mediated induction of Treg *in vitro*[155]. The authors therefore suggest that complex combinatorial or sequential

signals are involved in TGF-β-mediated Foxp3 induction. In turn, $CD25^+Foxp3^-$ effector T cells started to increase already from the very first day of co-culture, independent from whether microglia have been low-dose or high-dose activated (**Fig. 3.3**). Interestingly, kinetic analysis of both stimulation conditions exhibited a rapid increase of $CD25^+Foxp3^-$ T cells peaking on day 4, whereas $Foxp3^+$ Treg induction revealed a linear progression until day 7. Notably, also low-dose primed microglia induced a population of $CD25^+Foxp3^-$ effector T cells early during co-culture, before and while $Foxp3^+$ expression was increased. The subsequent questions, whether these $CD25^+Foxp3^-$ cells functionally differ from those induced under high-dose treatment, if they signal back on microglial cells thereby facilitating Treg conversion or proliferation, or if $CD25^+Foxp3^+$ arise from this population led us to analyze a set of cytokines produced during co-culture.

Monocyte chemoattractant protein-1 (MCP-1, CCL2), a member of the β-chemokine subfamily, was highly secreted in co-cultures when microglia were pre-exposed to IFNγ, with only minor changes in regard to low- or high-dose exposure (**Fig. 3.4**). Being involved in the activation and directional migration of leukocytes to inflammatory sites, early MCP-1 expression is reported in MS lesion[156], EAE parenchymal cells[157] or trauma brains[158]. A study on human fetal microglia revealed that IFNγ in concert with CD40-CD40L interaction induces MCP-1 expression in microglia mediated by the ERK1/2 MAPK pathway[159]. Because in our set-up IFNγ was removed from the system before $CD4^+$ T cell – microglia interaction (CD40-CD40L ligation), we suggest that a certain timeframe exists by which both signals can act in concert to induce MCP-1 secretion. Alternative sources of CNS MCP-1 are astrocytes and infiltrating monocytes[160,161]. There is emerging evidence, that activated microglia not only differentially affect the recruitment of Th1 and Th2 cells by secreting respective chemokines (MIP-1α versus MCP-1)[162], but also play a role in polarizing Th1 versus Th2 re-activation response by cytokine secretion, thereby potentially balancing the outcome of CNS immanent immune responses[71]. How MCP-1 influences $CD4^+$ T cell polarization is discussed by Luther and Cyster[163]. The authors summarize that MCP-1 ligation to its receptor CCR2 promotes Th2 effector cell development by acting on APCs (reduction of IL-12) as well as the $CD4^+$ T cell side (production of IL-4)[163]. Notably, we did not detect any IL-4 in our co-cultures, but large amounts of the Th2 cytokine IL-13 when stimulated with high-dose IFNγ/MOG (**Fig. 3.4**). The role of anti-inflammatory IL-13 on microglial cells *in vitro* was studied by Yang *et al*.[164] who reported, that IL-13 induces death of LPS activated microglia and suggested a Fas/FasL and NO independent mechanism. However, we did not observe significant changes in the number of microglia between the different co-culture conditions (data not shown). In addition, the IL-13 levels detected in high-dose treated co-cultures were 20-times less compared to the concentrations used by Yang and colleagues[164]. That microglia are

able to stimulate and re-stimulate Th2 responses, thereby contributing to a local regulatory circuit for the inhibition of Th1 responses has been shown before by the group of Francesca Aloisi[165]. However, it was surprising to find not only Th2 cytokines, but also Th1 (IFNγ) and Th17 (IL-17) cytokines being produced during culture of high-dose treated microglia with naive $CD4^+$ T cells. This led us to speculate about effector T cell plasticity specifically induced by microglia. In line with that, intracellular cytokine staining revealed IFNγ/IL-17 double-positive cells (**Fig. 3.5**). In sum, we observed a very distinct change in the effector T cell cytokine profile from co-cultures pre-treated with low-dose compared to high-dose IFNγ/MOG. The profile indicates high expression of IL-13, IL-17, IFNγ, IL-6 under high-dose conditions, while IL-23, IL-1β and IL-4 could not be detected at all. Mouse Th17 cell development in parallel with Foxp3 down-regulation mediated by IL-6 has been studied in detail[166]. Recent observations show that in the presence of IL-6, TGF-β is able to drive RORγt up-regulation and the subsequent induction of IL-17 cells. The contribution of TGF-β on Foxp3 and regulatory T cell induction as well as on Th17 cell induction depending on the microenvironment reflects the developmental plasticity of T helper cell responses. Recent reports suggest, that IL-23 is needed for terminal differentiation into Th17 cells as well as for their pathogenicity[167,168]. Taken together, high-dose IFNγ/MOG treatment induces a microglia phenotype, that mediates an effector T cell response that can not be simply assigned to one polarization, but resembles a mixture of Th1, Th2 and Th17 specific cytokines. In turn, low-dose co-cultures exhibiting high $Foxp3^+$ Treg levels, revealed expression of the above mentioned cytokines at very low levels or are completely absent, whereas IL-10 expression was increased. Both microglia and $Foxp3^+$ Treg cells are known to secrete anti-inflammatory IL-10, therefore a contribution of both to the IL-10 level cannot be ruled out.

In vivo different T helper cell subsets have been identified to contribute to the pathogenesis of EAE and MS[169]. Besides the Th1 phenotype, which was very potent in inducing EAE, also the adoptive transfer of *in vitro* generated Th17 or Th9 cells specific for MOG antigen was shown to induce CNS autoimmunity with different pathological phenotypes[170,171]. The specific cytokines produced by these distinct encephalitogenic T helper cell subsets might well account for pathological heterogeneity in MS patients[170]. Recently several modes of plasticity of T cell subsets have been described[172], including iTreg to Th17 and vice versa[173], Th17 to Th1[174] or Th2 to Th1[175]. Surprisingly, even under severe inflammatory conditions, such as MS disease or its animal model EAE, brain infiltrating Th cell subsets display substantial plasticity[169]. A recent study suggests that fate decisions of Th17 cells are shaped by different inflammatory conditions allowing them to switch from IL-17 to IFNγ production[176] in the chronic EAE model. Conversely, Th1 cells can also convert into IL-17/IFNγ double producers during the development of EAE, strongly questioning IL-17 expression as definition of an

end-stage T helper cell subset[177]. These data suggest that the tissue or cytokine milieu to which these cells migrate determines their final differentiation even after commitment to a specific T helper cell lineage.
Together with our own observations these reports strengthen the importance of the microenvironment on T cell differentiation, commitment and stability, especially when leukocytes enter an immunoprivileged site such as the CNS, where continuous re-activation is a critical step.

Two different approaches were applied to solve the question, whether elevated frequencies of Foxp3$^+$ cells result from proliferation of pre-existing Treg cells or from the conversion of naive precursors into *de novo* induced Foxp3$^+$ Treg cells. Using CD4$^+$CD25$^-$Foxp3$^-$ cells as starting population, Foxp3$^+$ cells could be readily detected after co-culture with differentially activated microglial cells indicating *de novo* induction of Treg cells from CD4$^+$ precursors (**Fig. 3.6**). However, Foxp3-negative regulatory T cells (iTr35[178], Tr1[179] or Th3 cells) are not depleted in this model. The frequency of Foxp3$^+$ cells in the *de novo* set-up was markedly reduced compared to co-cultures with total CD4$^+$. Because of that, we set up a second set of experiments analyzing proliferation of Treg (Foxp3$^+$) and effector T cells (Foxp3$^-$) from microglia co-cultured with total CD4$^+$ cells. Interestingly, in low-dose primed co-cultures only Foxp3$^+$ cells underwent proliferation, whereas Foxp3$^-$ cells did almost not (**Fig. 3.7**). In contrast, 6 days after co-culture high-dose treatment resulted in both, Foxp3$^-$ and Foxp3$^+$ cell proliferation to a similar extent. These data suggest that both, *de novo* induction and subsequent proliferation of Treg cells lead to accumulation of Foxp3$^+$ cells under low-dose conditions, indicating potentially different mechanisms by which microglia contribute to immune regulation. This is in line with *in vivo* and *in vitro* data from Kretschmer and colleagues, who reported that under “subimmunogenic” conditions (low-level antigen and lack of co-stimulation) peripheral DCs efficiently induce *de novo* Treg cells, but observed reduced conversion and high proliferation when highly activated DCs were present[105]. This view is further supported by the kinetic analysis of Foxp3$^+$ Treg frequencies over a 7 day period of co-culture. Already 3 days after being co-cultured with activated microglia, Foxp3$^+$ frequencies started to increase (**Fig. 3.3**), while proliferation was not observed (data not shown), suggesting conversion of naive into regulatory T cells at that specific time point. In a second step, cell proliferation was observed in low-dose stimulated co-cultures on day 5 (not shown) and 6, but restricted to the Foxp3$^+$ population. Again, high-dose stimulated microglia are found to induce extensive T cell proliferation but considerably less conversion into Foxp3$^+$ Treg, underlining the inverse relationship between proliferation and Treg conversion[105]. This also raised the question what factors drive proliferation exclusively in Foxp3$^+$ Tregs. Several studies describe IL-2 driven proliferation of Foxp3$^+$ regulatory T

cells[180–182]. While the IL-2 receptor (IL-2R) is expressed on mouse and human microglia[183], IL-2 itself is not produced by microglial cells. Activated, non-regulatory T cells are the cellular source of IL-2. One possible explanation is that the population of $CD4^+CD25^+Foxp3^-$ cells induced during the early days of co-culture (see days 1-4 of kinetic experiments), secrete IL-2, which than leads to expansion of *de novo* induced and pre-existing $Foxp3^+$ regulatory T cells. High frequencies of $Foxp3^+$ (resulting from *de novo* conversion) in low-dose activated co-cultures in turn suppress proliferation of $CD25^+Foxp3^-$ effector cells. Other studies link high CD86 expression on APC with Treg proliferation[184], that will be further discussed when microglial phenotypes are dissected. Notably, we did not detect any IL-4 in our set-up, excluding IL-4 driven Treg proliferation as documented by Xu *et al*.[185]. An additional explanation involves the signaling from *de novo* induced Treg back to the microglial cells[112,186].

The suppressive activity of microglia-induced Treg cells was proven *in vitro*, where sorted Foxp3-GFP^+ from low-dose co-cultures efficiently suppressed proliferation of encephalitogenic effector T cells up to a ratio of 1:50 (**Fig. 3.9**). To functionally test microglia-induced Treg in a clinical relevant model, we adoptively transferred $CD4^+CD25^+$ Treg cells purified from low-dose stimulated co-cultures into Bl6/J recipients one day before MOG immunization (**Fig. 3.10**). In this model of autoimmunity, autoantigen-specific T cells are primed in the periphery (draining LNs), infiltrate the CNS and result in progressive paralysis starting 10-12 days after immunization[187]. In contrast to PBS control mice and mice transferred with $CD4^+CD25^+$ cells (nTreg) isolated from the spleens of naive 2D2 mice, microglia-induced Treg ameliorated disease severity. The administration of microglia-induced Treg during priming did not delay onset or peak of disease, but significantly decreased disease severity and mortality rate, suggesting that microglia-induced Treg interfere with disease progression. Since Thomas Korn and colleagues found that CNS-derived autoantigen-specific effector T cells isolated at the peak of disease cannot be suppressed *in vivo* and *in vitro* by either CNS-derived or peripheral antigen-specific Treg cells, disease amelioration mediated by transfer of microglia-induced Treg might already take place in the periphery (draining LNs). Surprisingly, in our study nTreg isolated from naive MOG TCR transgenic animals failed to suppress EAE disease scores. This is in apparent contrast to findings of Kohm *et al.*, who reported significant protection from the development of clinical EAE in Bl6/J mice when transferring $CD4^+CD25^+$ nTreg fom WT animals[115]. However, the authors transferred 2×10^6 cells, a considerable greater number of cells than used in our experimental set-up (500.000 cells per mouse). To rule out the potential effect of different TCR specificity in both set-ups, we included nTreg from BL6/J WT animals in active EAE, but observed no EAE disease protection in control to PBS treated mice (data not shown). This

data-set illustrates the potential of relatively small numbers of microglia-primed, MOG-specific Treg to ameliorate MOG EAE.

How epigenetic processes regulate cell lineage differentiation has already been shown for Th1 and Th2 cells, where histone and covalent DNA modifications lead to converse pattern of gene activation and silencing[188]. Recent studies now identified a Treg-specific demethylated region (TSDR) within the murine and human *foxp3* locus, with functional importance for long-term stability of regulatory T cells[126,124]. The authors reported an almost complete demethylation of this conserved region in natural occurring Treg cells, while TGF-β induced Treg showed only weakly demythylated CpG motifs[126]. Their finding correlated with the loss of suppressive capacities and Foxp3 expression when TGF-β was removed from these cells, indicating an unstable Treg identity. We therefore purified $CD4^+CD25^+$ cells from low-dose primed microglia - $CD4^+$ T cell co-cultures and analyzed their DNA methylation pattern in the TSDR of *foxp3*. Compared to $CD4^+CD25^+$ nTreg isolated from lymph nodes of Bl6/J WT animals, microglia-induced Treg showed a very similar methylation pattern for the selected amplicons, indicating a comparably stable phenotype. Notably, the underlying Treg purification method based on CD25 expression resulted in $CD25^+$ cells containing a distinct proportion of $Foxp3^-$ cells ($Foxp3^+$: $Foxp3^-$ 1:1). Therefore, nTreg control cells were applied in the same ratio. Both samples were found within the same range of methylation in all tested CpG motifs, which is approximately 50% (**Fig. 3.8**). In absolute terms, this implies that all $Foxp3^+$ cells in these samples should have been completely unmethylated. Keeping in mind, that around two thirds of all $Foxp3^+$ Treg cells, which arise from total $CD4^+$ cells, are *de novo* induced, this would account for a predominantly unmethylated *foxp3* locus in microglia-induced Treg indicating a stable Treg lineage identity. Together with the results from *de novo* induction and proliferation studies, this particular set-up demonstrates the need to analyze newly induced and proliferation-derived $Foxp3^+$ Treg from microglia co-cultures as separate pools.

4.3.2 The APC side – Microglia antigen presentation is tightly regulated

The above discussed differential $CD4^+$ T cell differentiation, cytokine patterns and suppressive potential in response to distinct microglia activation prompted us to further study microglia phenotypes related to antigen presentation. We hypothesized that the distinct levels of stimuli that were applied to activate microglia result in different microglia phenotypes, which than directly polarize $CD4^+$ T cell responses. Although the difference of respective IFNγ/MOG concentrations, namely 10 U/ml / 1 µg/ml *versus* 100 U/ml / 10 µg/ml, does not seem to be that high, we have to keep in mind that microglia, the immune sentinels of the brain, rapidly undergo changes in morphology or function even in response to poor

stimuli. How the distinct phenotypes differ in terms of surface molecule expression or cytokine production was central in this part of the project.

The concept of “classical/M1” (IFNγ and LPS mediated) versus “alternative/M2” (IL-4 and IL-13 mediated) activation of peripheral tissue macrophages has been critically discussed in relation to macrophage heterogeneity and differentiation[189]. In addition, “regulatory” macrophages (IFNγ mediated) that suppress T cell proliferation have been described for mouse[190] and human[191]. Weber *et al*. demonstrated that glatiramer acetate treatment generated anti-inflammatory type II monocytes that directed differentiation of Th2 and Foxp3$^+$ Treg cells[106]. Whether such classification also exists for microglia and how this would affect homeostatic immune responses or responses to brain injury or disease is still not known[192]. To work out which activation-dependent factors influence microglia-mediated T cell differentiation, we analyzed microglial proteins providing signal 1-3.

4.3.3 Signal 1 – TCR-MHCII-Ag complex

In contrast to professional APC that constitutively express MHC class II, such as B lymphocytes, dendritic cells or other cells of the monocyte-macrophage lineage, microglia are MHCII negative in their resting state, but can immediately up-regulate MHCII in response to injury or disease. The up-regulation of microglial MHCII in response to type II interferon (IFNγ) responsive genes has been described in many studies[146,154]. The observed IFNγ dose-dependency of MHCII expression (**Fig. 3.11**) is in line with other studies on *per se* MHCII-negative cell types, such as mesenchymal stromal cells[193] or epithelial cells[194]. Most notably, raising IFNγ concentrations up to 1000 U/ml did not shift all microglial cells to MHCII$^+$. Instead a population of around 40% remained MHCII$^-$, independent of the amount of IFNγ applied (data not shown), supporting the idea of microglia functional heterogeneity[195]. This is consistent with other reports stating that even upon full activation, the levels of MHCII and costimulatory molecules are much lower than that seen of professional, peripheral APC[7].

Facing the point that adult microglia might show a slightly different responsiveness than that of postnatal derived cells[196], and that microglial expression of MHCII is known to increase in normal aging brains[197,198] we contrasted our findings from postnatal microglia with experiments performed with microglia directly isolated from adult brains. The observed marginal trend in MHCII expression between low- and high-dose stimulated adult microglia (**Fig. 3.21**) is in apparent contrast to the situation in postnatal microglia. However, we did not follow these results since the protocol for establishing adult microglia cultures involves the use of L929 conditioned media, which contains MCSF and other possibly unknown growth factors or cytokines that can potentially influence IFNγ-mediated activation and subsequently CD4$^+$ T cell differentiation.

In vivo MHCII expressing microglia have been implicated in a number of injury or disease settings, such as AD[39,40], MS[46], CNS injury[58,199] or stroke[200,201], underlining their potential role in the reactivation of CNS infiltrating T cells. The potential of microglia to induce antigen-specific Foxp3$^+$ regulatory T cells in an MHCII-dependent way has not been studied so far. Using Middle Cerebral Artery Occlusion (MCAO) as a model for ischemic stroke our group previously demonstrated a strong accumulation of Foxp3$^+$ Treg cells in the ischemic hemisphere accompanied by the elevated presence and activation of microglia[201]. Similarly, using ECL, which models a steril, non-inflammatory brain lesion, we could also show an increase in the frequency of CNS Foxp3$^+$ Treg cells peaking 14 days after the insult (Stubbe, unpublished). Immunohistochemistry identified MHCII$^+$Iba1$^+$ cells in contact or close proximity to Foxp3$^+$ Treg cells in the lesioned hemispheres (Stubbe, unpublished). Following this first hints, we addressed the question whether CNS MHCII expression is required for accumulation of Foxp3$^+$ Tregs cells. When subjecting MHCII$^{-/-}$ mice to ECL, we observed CD4$^+$ T cell infiltration on a negligible background level compared to WT mice, without significant differences between ipsi- and contralateral hemispheres (**Fig. 3.23**), suggesting that the initial, peripheral T cell activation is fundamental for overall CD4$^+$ T cell invasion into the CNS. Therefore we intended to generate bone marrow chimeras with MHCII expression restricted to either peripheral or CNS resident APC, subject these mice to ECL and analyze the presence of CNS Foxp3$^+$ Treg cells in respective animals (**Fig. 3.24**, **Fig. 3.26**). While the generation of WT → WT and WT → MHCII$^{-/-}$ chimeras succeeded (**Fig. 3.25**), MHCII$^{-/-}$ → WT chimeric mice developed symptoms attributed to radiation-induced colitis as described by Marguerat *et al*. 8 weeks after reconstitution[134]. The authors suggested that the gamma-irradiation induced damage to the intestinal epithelium facilitates the transfer of gut luminal antigens to the lamina propria followed by massive lymphocyte activation that is normally controlled by regulatory T cells. According to them, reconstituting gamma-irradiated mice with cells of hematopoietic origin that lack MHC class II prevents induction and/or activation of regulatory T cells and results in fatal colitis[134]. This view is further supported by other studies proving that Treg depletion produces inflammatory bowel disease even without irradiation, which likely results from excessive immune responses to commensal bacteria in the intestine[202]. We therefore developed two different strategies to overcome this fatal progression; continuous antibiotic treatment to limit bacterial invasion and adoptive Treg therapy from congenic animals (Thy1.1) on the day of reconstitution. Independent of the treatment applied, all MHCII$^{-/-}$ → WT chimeras succumbed the symptoms of inflammatory bowel disease. Since the reasons for symptom development in these mice are largely unclear, we can only speculate about failing with the Treg transfer strategy. Possible explanations involve small numbers of transferred cells (e.g. 7 x 10^5 Treg cells/mouse, while

a normal mouse contains usually 2.5-3 x 10^6 Treg[115]), missing activation/proliferation or the need for antigen-specific Tregs locally at the site of inflammation.

Analyzing numbers and composition of CNS infiltrating leucocytes in WT → WT and WT → MHCII$^{-/-}$ chimeras 14 days after ECL surgery revealed that, while overall leucocyte infiltration was constant (#CD45^{high} cells, **Fig. 3.27 a**), the number of CD4$^+$ cells recruited to the lesioned site as well as the ratio of Foxp3$^+$ Treg per infiltrated CD45^{high} cell was significantly diminished in WT → MHCII$^{-/-}$ mice lacking MHCII specifically in the CNS (**Fig. 3.27 b, d**). MHCII expression on CNS resident cells might therefore account for elevated Treg cell numbers *in vivo.* However, a direct comparison of ipsilateral Foxp3$^+$ Treg frequencies in the CD4$^+$ compartment turned out to be misleading, since elevated Treg frequencies were found in blood and CLN of WT → MHCII$^{-/-}$ chimeras compared to WT → WT mice. Because our statement that Foxp3$^+$ regulatory T cells accumulate in the ischemic hemisphere after stroke[201] and also in the lesioned hemisphere after cortex lesion (Stubbe, unpublished) was based on finding significantly increased Foxp3$^+$ frequencies of the CD4$^+$ population in the ipsilateral hemispheres compared to contralateral hemispheres, peripheral lymphatics and the blood, we were surprised to find that the significant difference in Treg frequency between ipsilateral hemisphere and brain draining, cervical lymph nodes observed in WT (data not shown) and WT → WT chimeric mice was not detectable in WT → MHCII$^{-/-}$ chimeric animals. A possible explanation for the elevated peripheral Tregs level might come from studies describing “homeostatic” Treg proliferation, such as lymphopenia-induced proliferation when Treg cells are adoptively transferred into lymphocyte-deficient hosts[203–205]. Since homeostatic Treg proliferation is shown to require self-MHC class II complexes[204], we speculate whether the reconstitution of MHCII$^{-/-}$ mice with MHCII potent cells of hematopoetic origin favors the niche for Treg cells. Given the concept of a fixed peripheral niche for Treg cells[203] that allows them to expand until a certain population size independently of naive CD25$^-$ T cells in a model of adoptive cell transfer to CD3ε$^{-/-}$ recipients[206], this set of data suggests the contribution of MHCII expressing non-hematopoetic cells on regulating Treg homeostasis in the periphery.

However, there are several points concerning this particular experimental set up that have to be critically addressed. Firstly, there is an ongoing debate whether and to what extend microglia in the adult brain are replenished by blood circulating progenitor cells[207]. To control for the lack or presence of MHCII expressing newly recruited microglia, markers would have been necessary that allow for the discrimination between both populations. Secondly, there are hints that gamma irradiation can impair BBB integrity[208]. This might lead to invasion of MHCII potent monocytes and also other immune cells even before subjecting the animals to ECL. However, we did not detect significant differences in the number of MHCII expressing CD45^{high}CD11b$^+$ CNS infiltrating monocytes/macrophages between WT and MHCII chimeras.

In addition, there are a few other CNS resident cell types that can contribute to CNS MHCII expression, namely perivascular macrophages, pericytes and astrocytes that have been shown to up-regulate MHCII under severe inflammatory conditions[209,210]. Lastly, MHCII bone marrow chimeras represent a complex and artificial model, that on its own needs further immunological clarification[134] before even more complicated cortex surgeries are evaluated based on the current knowledge. In sum, the data generated in MHCII bone marrow chimeric mice do not conclusively demonstrate the role of microglial MHCII expression for CNS Treg levels *in vivo*.

4.3.4 Signal 2 – Co-stimulatory molecules

The differential regulation of co-stimulatory molecules is an inherent part of the concept of macrophage polarization[211], but has not been applied so far to more specifically illuminate the diverse activation modes of microglial cells in the CNS.

IFNγ is known to enhance microglial CD40 expression in mice[212,213] and men[159,214]. Ponomarev *et al.* described a two-step activation of microglia during EAE, at the onset and at the peak of disease, with only the second activation step being CD40-dependent[48]. Using CD40 bone marrow chimeric mice they demonstrated that CD40 expression by microglia contributes to proliferation of encephalitogenic T cells in the CNS and therefore effects disease progression. Interestingly, they describe two populations of MHCII expressing microglia during the peak of EAE; $CD45^{high}$ activated microglia expressing also high levels of co-stimulatory CD40 and CD86, and "resting" $CD45^{low}$ microglia expressing very low levels of CD40 and CD86 what they define as bystander activation. As a functional role for bystander activated microglia they suggest down-modulation of CNS inflammation by mediating tolerance[46,48].

In line with other reports we found low-dose activated microglia to express intermediate levels of CD40 and further IFNγ dose-dependent up-regulation of CD40 mRNA (**Fig. 3.14**)[212]. Since recent studies suggested anti-CD40L mAb treatment to selectively inhibit proliferation of effector T cells, but not $CD4^+CD25^+$ Treg cells, we performed high-dose co-cultures in the presence of anti-CD154[129]. Notably, we detected around 38% of CD25 expressing $CD4^+$ cells, but no increase of $Foxp3^+$ Treg cells in those co-cultures, indicating that $CD4^+$ T cell activation was affected, but not impaired and that blocking CD40-CD40L signaling does not increase Treg induction mediated by microglial cells.

Microglia favoring the induction of regulatory T cells revealed an intermediate level of CD86, whereas CD80 expression was shown not to be regulated (**Fig. 3.14**). Performing ECL surgery on rats, CD80 and CD86 regulation on microglia in response to mechanically induced axonal degeneration was studied by the Bechmann group. They could show that after ECL, myelin is presented in a MHCII/CD86 rather than in a MHCII/CD80 context,

leading to potentially protective autoimmunity[58]. This is in line with our *in vitro* findings showing that the different IFNγ/MOG stimulations applied to microglial cells did not lead to changes of CD80 mRNA levels. On the contrary, using MCAO to model ischemic stroke Gelderblom *et al.* detected an ipsilateral increase in microglial CD80 expression 3 days after the insult[215]. Their findings were further extended and discussed by Felger *et al.* who reported a strong increase of CD80 relative to CD86 but on brain infiltrating and resident DCs[147]. During severe CNS inflammation, e.g. in MS disease, CD80 seems to be the predominant co-stimulatory molecule, detected in lesion sites and cerebrospinal fluid of patients[216,217], pointing towards a role for CD80 co-stimulation in a rather inflammatory situation. These findings illustrate the model dependent activation on brain resident APC and the need to carefully distinguish between the different CNS resident APC population. Negative co-stimulatory molecules, such as Programmed cell death ligand 1 (PD-L1) or DC-SIGN/CD209, have also been associated with distinct macrophage activation, e.g. IL-10 producing regulatory macrophages[190,218,219]. In addition, a few studies demonstrated upregulated PD-L1 expression *in vivo* in EAE brains and IFNγ-inducible expression on microglia[220,221]. However, these molecules were not found to be regulated on microglial mRNA level in response to the established IFNγ/MOG stimulations (data not shown). In sum, we could correlate activation-dependent differences on T cell polarization with distinct regulation of the co-stimulatory molecules CD40 and CD86 on microglial cells. Reduced levels of these co-stimulatory molecules have also been described for anti-inflammatory type II monocytes[106] and type II-activated macrophages[222], both revealing suppressive effects on EAE disease.

4.3.5 Signal 3 – Microenvironment

Locally secreted cytokines, which reflect the inflammatory or anti-inflammatory micromilieu, are thought to be the major determinant of CD4 T cell differentiation and conversion. As T cells, macrophages can also polarize to distinct phenotypes (M1 *vs.* M2a, M2b, M2c) secreting discrete sets of cytokines and chemokines and thereby shaping the local cytokine milieu. In microglia, Häusler and colleagues showed that IFNγ dose-dependently modulated LPS-induced cytokine and chemokine release *in vitro*, indicating a potent and complex immune modulatory effect of IFNγ on microglial cells[223]. Based on their findings the authors speculated about the influence of differential IFNγ stimulation on polarization of Th1/Th2 responses *via* microglial cytokine and chemokine synthesis[223].

As a major difference between microglia mediating Foxp3$^+$ Treg induction and those directing CD4 T cells towards effector cells, we found immunosuppressive IL-10 to be up-regulated exclusively in low-dose primed microglia (**Fig. 3.16**). This might point towards an alternative activation of microglial cells, similar to type II monocytes[106] and alternatively activated

macrophages[224], which are characterized by increased IL-10 production. Because macrophages are known to be a source as well as a target for anti-inflammatory IL-10, we wanted to further clarify whether the presence of IL-10 during microglia priming has any impact on the $CD4^+$ T cell response. Demonstrating that the pre-treatment of microglia with high-dose IFNγ/MOG in combination with recombinant IL-10 rescues the regulatory phenotype and leads to increased $Foxp3^+$ Treg induction (**Fig. 3.17**), we speculated that anti-inflammatory IL-10 modulates IFNγ responsive genes that contribute to microglial antigen presentation. As positive control, we analyzed levels of inducible NO synthase (iNOS), which is known to be down modulated by IL-10 in macrophages[225]. Surprisingly, levels of the co-stimulatory molecules CD40, CD80 and CD86 were unchanged between high-dose and high-dose + IL-10 stimulated microglia (**Fig. 3.18**), indicating that the factors or the combination of factors making up the regulatory microglia phenotype have not been sufficiently identified.
Whether tissue macrophages differentiated in a certain microenvironment are terminally differentiated or can respond with altering their phenotype to local changes was debated for a long time, but recent studies now suggest that macrophage activation states are reversible[226]. We therefore performed experiments where exogenous IL-10 was applied 24 h after microglia were stimulated with high-dose and observed a similar rescue of the regulatory microglia phenotype (data not shown). This led us to speculate that the polarization of microglial cells by high levels of IFNγ is reversible by anti-inflammatory signals, such as IL-10 and therefore does not represent and end-stage differentiation. To further examine the role of microglial IL-10 on Treg induction we generated primary microglia from IL-10 deficient mice and applied them in the co-culture assay. Even though repeated many times, we were not able to generate consistent data on microglia mediated $Foxp3^+$ Treg induction (data not shown). Notably, cytokine analysis revealed that IL-10KO microglia secreted far more TNFα, IL-6, MCP-1 and CXCL1/KC than WT microglia independent from the stimulus (data not shown), questioning the suitability of these mice to assess the contribution of IL-10 on its own.

Numerous studies argue that IFNγ stimulation favors macrophage differentiation toward an M1 profile[227]. Since microglia populate an immunologically hostile environment, they face stimuli clearly distinct from those, confronted by peripheral macrophages. In the healthy CNS they constantly monitor their environment and adjacent cells and continuously integrate signals from neurons and other glial neighbors[228]. We therefore assume that microglia are able to respond a lot more sensitive to IFNγ danger signals than peripheral, professional APC. This is consistent with a study by Hu *et al.* who demonstrated that different populations of monocytes/macrophages have different sensitivities to IFNγ stimulation[229]. Based on our

results we suggest a fine-tuned mechanism by which microglia regulate proteins needed for efficient antigen presentation and secretion of cytokines/chemokines depending on their activation state and microenvironment (**Fig. 4.1**). This work supports the view of diverse microglia subtypes balancing inflammation through differential effects on effector versus regulatory T cells, suggesting an essential role of microglia in regulating immune activation in the CNS.

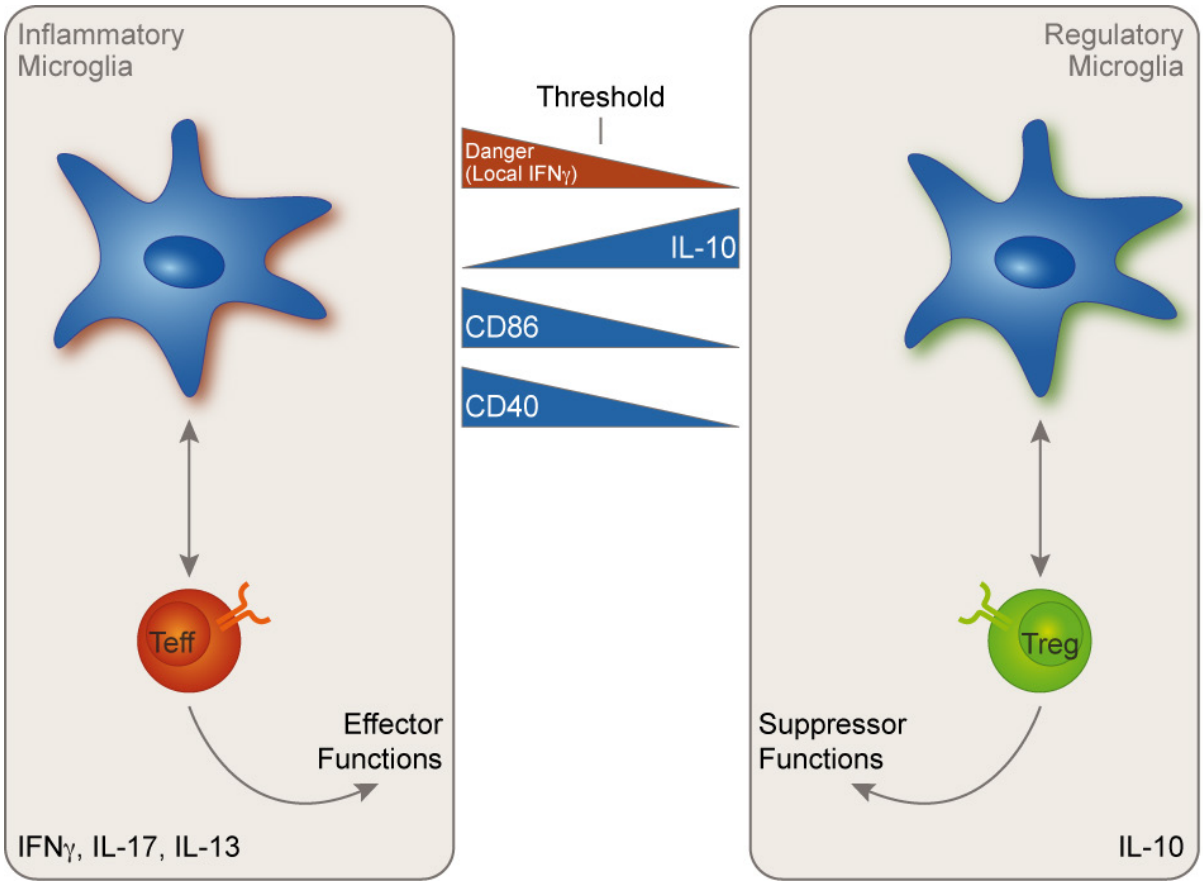

Fig. 4.1 Model of inflammatory versus regulatory microglia activation.
When confronted with IFNγ signals above a certain threshold, microglia aquire an inflammatory phenotype characterized by high levels of co-stimulatory molecule expression (CD40, CD86) and low amounts of IL-10, and induces effector T cells (Teff) with a pro-inflammatory cytokine profile. A regulatory microglia phenotype results from IFNγ danger signals below a certain threshold, is characterized by increased microglial IL-10 production and low levels CD40 and CD86 and induces $CD4^+$ regulatory T cells (Treg) with suppressive functions.

4.4 Conclusions and Outlook

It is now evident that CNS resident microglia, rather than simply proceeding from “resting” to “activated” states, display various modes of activation with diverse functional outcomes. Whether they exert a beneficial or destructive effect on the CNS tissue depends on communication signals from neurons, surrounding glial cells or infiltrating immune cells and from the micromilieu confronted with[14].

We speculate that microglia, depending on their specific activation, contribute to regulation of local $CD4^+$ T cell responses. The here performed *in vitro* studies revealed that microglia react very sensitive to the inflammatory type II interferon IFNγ by modulating molecule levels for efficient antigen presentation and levels of secreted cytokines and chemokines. We could further show that their distinct activation favored either Treg or effector T cell responses *in vitro*. The Treg development mediated by low-dose IFNγ/MOG stimulated microglia was shown to be an interplay of *de novo* induced Treg and proliferation of pre-existing nTreg cells (**Fig. 3.6**, **Fig. 3.7**). Previously, our group not only demonstrated reduced EAE severity in cortex lesioned mice[119], but also the relatively late accumulation of Treg cells in mice subjected to cortex lesion (14-30 days after the insult, T. Stubbe, personal communication). Whether or not CNS inflammation can be controlled by the presence of Treg cells probably will depend on the inflammatory cytokine milieu. We assumed that ECL and also partially MCAO model particular weak inflammatory CNS injuries, where potentially low IFNγ levels from the initial invasion of T and NK cells give rise to microglia with a regulatory phenotype. In these settings, newly recruited T cells, which require continuous antigen-specific re-activation would then encounter microglia directing them towards tolerance. This might also explain the late and long-lasting accumulation of Treg cells after experimental ECL and MCAO which is correlated with a significant accumulation of MHCII expressing microglia ($CD45^{dim}CD11b^+$ cells) in the ipsilateral hemispheres[201].

Importantly, studies documenting T cell plasticity in the CNS[169,176] let us to question final T cell commitment after having crossed the barriers, especially in a weak inflammatory model such as ECL, and we hypothesized that T cell re-activation can be shaped by microglial antigen presentation and the local cytokine milieu. We already demonstrated that infiltrating $Foxp3^+$ Treg can proliferate locally in the CNS[201] in a model of stroke. Here we show that microglia have the potential to mediate *de novo* induction and proliferation of $Foxp3^+$ Treg. Therefore, subimmunogenic activation of microglia by low-level IFNγ could be a possible regulatory mechanism that limits the expansion of autoantigen-specific effector T cells in the CNS. Such mechanisms could explain why brain injury, despite the presence of myelin-specific T cells in injured regions, does not lead to destructive autoimmunity.

The control of microglial activation under pathological conditions seems to be a promising therapeutic target for limiting CNS inflammation. The fact that microglia show distinct, activation-dependent phenotypes leads to the question how we can take advantage of neuroprotective microglial activation, without promoting neurodestructive processes. For MS disease, where the re-activation of infiltrating T cells behind the brain barriers is a critical point in the initial phase of disease, the strength of re-activation has been suggested to determine the degree of subsequent parenchymal inflammation[15,230]. Given the fact that the strength of re-activation depends on both the affinity of the particular TCR for its cognate antigen and on the number of specific antigen-MHC complexes available on the surface of APC[15], the fine-tuning of regulating antigen presentation is of particular importance for cells that are *per se* MHCII negative. Since the co-stimulatory pathways have recently turned out to be of bilateral signaling influencing also the phenotype of the APC[218], much more effort is needed to better understand the interaction of microglia and the very first infiltrating T cells. Furthermore, regulatory microglia need to be characterized in more detail with focusing on the molecular switches and signaling pathways that drive their phenotypic conversion. Recently, the Notch pathway was identified to determine M1 *versus* M2 macrophage polarization[231]. Our IL-10 rescue studies provide first hints, that the molecular factor/factors that allows them to induce Foxp3$^+$ Treg cells is/are somehow influenced by IL-10 signaling. How exactly IL-10 modifies IFNγ induced activation of microglia should be analyzed for a wider spectrum of genes and/or proteins, e.g. by comparing low-dose, high-dose and high-dose+ IL-10 activated microglia on a microarray.

Of overall importance is the identification of a microglia specific marker that allows the clear differentiation between newly recruited monocytes/macrophages and CNS resident microglia for flow cytometry and immunohistochemistry. In addition, *in vivo* approaches with the aim to identify location, persistence and motility of regulatory microglia would deepen our understanding of local CNS immune regulation.

Going back to the initial experiments of our group, where ECL-lesioned mice were subjected to EAE immunization and showed reduced disease severity compared to sham operated animals[119], further studies could use conditional Foxp3 knockout mice to prevent accumulation of Foxp3$^+$ Treg after ECL and immunize the mice as soon as peripheral Treg levels are restored, to evaluate the functional relevance of CNS Treg cells.

5 References

1. Shrikant, P. & Benveniste, E. N. The central nervous system as an immunocompetent organ: role of glial cells in antigen presentation. *J Immunol* **157,** 1819–1822 (1996).
2. Overview of Wound Healing in Different Tissue Types - Indwelling Neural Implants - NCBI Bookshelf. at <http://www.ncbi.nlm.nih.gov/books/NBK3938/>
3. Chen, Y. & Liu, L. Modern methods for delivery of drugs across the blood–brain barrier. *Advanced Drug Delivery Reviews* **64,** 640–665 (2012).
4. Hickey, W. F. Basic principles of immunological surveillance of the normal central nervous system. *Glia* **36,** 118–124 (2001).
5. Bechmann, I. *et al.* Immune surveillance of mouse brain perivascular spaces by blood-borne macrophages. *European Journal of Neuroscience* **14,** 1651–1658 (2001).
6. Hickey, W. F., Hsu, B. L. & Kimura, H. T-lymphocyte entry into the central nervous system. *J. Neurosci. Res.* **28,** 254–260 (1991).
7. Carson, M. J., Doose, J. M., Melchior, B., Schmid, C. D. & Ploix, C. C. CNS immune privilege: hiding in plain sight. *Immunol Rev* **213,** 48–65 (2006).
8. Raivich, G. *et al.* Neuroglial activation repertoire in the injured brain: graded response, molecular mechanisms and cues to physiological function. *Brain Research Reviews* **30,** 77–105 (1999).
9. Schluesener, H. J. & Wekerle, H. Autoaggressive T lymphocyte lines recognizing the encephalitogenic region of myelin basic protein: in vitro selection from unprimed rat T lymphocyte populations. *J. Immunol.* **135,** 3128–3133 (1985).
10. Increased frequency of interleukin 2-responsive T cells specific for myelin basic protein and proteolipid protein in peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. *J Exp Med* **179,** 973–984 (1994).
11. Cserr, H. F. & Knopf, P. M. Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. *Immunol. Today* **13,** 507–512 (1992).
12. Engelhardt, B. T cell migration into the central nervous system during health and disease: Different molecular keys allow access to different central nervous system compartments. *Clinical and Experimental Neuroimmunology* **1,** 79–93 (2010).
13. Engelhardt, B. & Ransohoff, R. M. The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. *Trends in Immunology* **26,** 485–495 (2005).
14. Schwartz, M., Shaked, I., Fisher, J., Mizrahi, T. & Schori, H. Protective autoimmunity against the enemy within: fighting glutamate toxicity. *Trends in Neurosciences* **26,** 297–302 (2003).
15. Goverman, J. Autoimmune T cell responses in the central nervous system. *Nat Rev Immunol* **9,** 393 (2009).
16. Baumann, N. & Pham-Dinh, D. Biology of Oligodendrocyte and Myelin in the Mammalian Central Nervous System. *Physiol Rev* **81,** 871–927 (2001).
17. Jacobson, S. & Marcus, E. M. *Neuroanatomy for the Neuroscientist*. (Springer, 2011).
18. Fünfschilling, U. *et al.* Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature* **485,** 517–521 (2012).
19. Wekerle, H. & Sun, D. Fragile privileges: autoimmunity in brain and eye. *Acta Pharmacologica Sinica* **31,** 1141–1148 (2010).

20. Galoyan, A. & Lajtha, A. *Handbook of Neurochemistry and Molecular Neurobiology: Neuroimmunology*. (Springer, 2007).
21. Streit, W. J., Graeber, M. B. & Kreutzberg, G. W. Functional plasticity of microglia: A review. *Glia* **1,** 301–307 (1988).
22. Giulian, D. & Vaca, K. Inflammatory glia mediate delayed neuronal damage after ischemia in the central nervous system. *Stroke* **24,** I84–90 (1993).
23. Lawson, L. J., Perry, V. H., Dri, P. & Gordon, S. Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. *Neuroscience* **39,** 151–170 (1990).
24. Saijo, K. & Glass, C. K. Microglial cell origin and phenotypes in health and disease. *Nature Reviews Immunology* **11,** 775–787 (2011).
25. Chan, W. Y., Kohsaka, S. & Rezaie, P. The origin and cell lineage of microglia—New concepts. *Brain Research Reviews* **53,** 344–354 (2007).
26. Nimmerjahn, A., Kirchhoff, F. & Helmchen, F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science* **308,** 1314–1318 (2005).
27. Gabrusiewicz, K. *et al.* Characteristics of the Alternative Phenotype of Microglia/Macrophages and its Modulation in Experimental Gliomas. *PLoS ONE* **6,** e23902 (2011).
28. Kettenmann, H., Hanisch, U.-K., Noda, M. & Verkhratsky, A. Physiology of Microglia. *Physiological Reviews* **91,** 461 –553 (2011).
29. Bessis, A., Béchade, C., Bernard, D. & Roumier, A. Microglial control of neuronal death and synaptic properties. *Glia* **55,** 233–238 (2006).
30. Ransohoff, R. M. & Cardona, A. E. The myeloid cells of the central nervous system parenchyma. *Nature* **468,** 253–262 (2010).
31. Cardona, A. E. *et al.* Control of microglial neurotoxicity by the fractalkine receptor. *Nature Neuroscience* **9,** 917–924 (2006).
32. Constam, D. B. *et al.* Differential expression of transforming growth factor-beta 1, -beta 2, and -beta 3 by glioblastoma cells, astrocytes, and microglia. *J. Immunol.* **148,** 1404–1410 (1992).
33. Frei, K., Lins, H. & Fontana, A. Production and function of IL-10 in the central nervous system. *Schweiz Arch Neurol Psychiatr* **145,** 30–31 (1994).
34. Olson, J. K. & Miller, S. D. Microglia Initiate Central Nervous System Innate and Adaptive Immune Responses through Multiple TLRs. *J Immunol* **173,** 3916–3924 (2004).
35. Rock, R. B. *et al.* Role of Microglia in Central Nervous System Infections. *Clin Microbiol Rev* **17,** 942–964 (2004).
36. Lehnardt, S. Innate immunity and neuroinflammation in the CNS: The role of microglia in Toll-like receptor-mediated neuronal injury. *Glia* **58,** 253–263 (2010).
37. Weiner, H. L. & Frenkel, D. Immunology and immunotherapy of Alzheimer's disease. *Nat Rev Immunol* **6,** 404–416 (2006).
38. Rogers, J. & Lue, L.-F. Microglial chemotaxis, activation, and phagocytosis of amyloid β-peptide as linked phenomena in Alzheimer's disease. *Neurochemistry International* **39,** 333–340 (2001).
39. Schenk, D. *et al.* Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature* **400,** 173–177 (1999).

40. Shaftel, S. S. *et al.* Sustained hippocampal IL-1β overexpression mediates chronic neuroinflammation and ameliorates Alzheimer plaque pathology. *Journal of Clinical Investigation* **117,** 1595–1604 (2007).
41. Lassmann, H. Neuropathology in Multiple Sclerosis: New Concepts. *Mult Scler* **4,** 93–98 (1998).
42. Sospedra, M. & Martin, R. Immunology of Multiple Sclerosis*. *Annual Review of Immunology* **23,** 683–747 (2005).
43. Weiner, H. L. & Selkoe, D. J. Inflammation and therapeutic vaccination in CNS diseases. *Nature* **420,** 879–884 (2002).
44. Domingues, H. S., Mues, M., Lassmann, H., Wekerle, H. & Krishnamoorthy, G. Functional and Pathogenic Differences of Th1 and Th17 Cells in Experimental Autoimmune Encephalomyelitis. *PLoS One* **5,** (2010).
45. Dittel, B. N. CD4 T cells: Balancing the coming and going of autoimmune-mediated inflammation in the CNS. *Brain, Behavior, and Immunity* **22,** 421–430 (2008).
46. Ponomarev, E. D., Shriver, L. P., Maresz, K. & Dittel, B. N. Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. *Journal of Neuroscience Research* **81,** 374–389 (2005).
47. Rasmussen, S. *et al.* Persistent Activation of Microglia Is Associated with Neuronal Dysfunction of Callosal Projecting Pathways and Multiple Sclerosis-Like Lesions in Relapsing–Remitting Experimental Autoimmune Encephalomyelitis. *Brain* **130,** 2816–2829 (2007).
48. Ponomarev, E. D., Shriver, L. P. & Dittel, B. N. CD40 Expression by Microglial Cells Is Required for Their Completion of a Two-Step Activation Process during Central Nervous System Autoimmune Inflammation. *J Immunol* **176,** 1402–1410 (2006).
49. Heppner, F. L. *et al.* Experimental autoimmune encephalomyelitis repressed by microglial paralysis. *Nature Medicine* **11,** 146–152 (2005).
50. Prass, K. *et al.* Stroke-induced Immunodeficiency Promotes Spontaneous Bacterial Infections and Is Mediated by Sympathetic Activation Reversal by Poststroke T Helper Cell Type 1–like Immunostimulation. *J Exp Med* **198,** 725–736 (2003).
51. Brait, V. H., Arumugam, T. V., Drummond, G. R. & Sobey, C. G. Importance of T lymphocytes in brain injury, immunodeficiency, and recovery after cerebral ischemia. *Journal of Cerebral Blood Flow & Metabolism* **32,** 598–611 (2012).
52. Macrez, R. *et al.* Stroke and the immune system: from pathophysiology to new therapeutic strategies. *The Lancet Neurology* **10,** 471–480 (2011).
53. Yenari, M., Kauppinen, T. & Swanson, R. Microglial activation in stroke: Therapeutic targets. *Neurotherapeutics* **7,** 378–391 (2010).
54. Kwidzinski, E. *et al.* Self-tolerance in the immune privileged CNS: lessons from the entorhinal cortex lesion model. *J. Neural Transm. Suppl* 29–49 (2003).
55. Fujita, T., Yoshimine, T., Maruno, M. & Hayakawa, T. Cellular Dynamics of Macrophages and Microglial Cells in Reaction to Stab Wounds in Rat Cerebral Cortex. *Acta Neurochirurgica* **140,** 275–279 (1998).
56. Fitch, M. T., Doller, C., Combs, C. K., Landreth, G. E. & Silver, J. Cellular and Molecular Mechanisms of Glial Scarring and Progressive Cavitation: In Vivo and In VitroAnalysis of Inflammation-Induced Secondary Injury After CNS Trauma. *J. Neurosci.* **19,** 8182–8198 (1999).

57. Hailer, N. P., Bechmann, I., Heizmann, S. & Nitsch, R. Adhesion molecule expression on phagocytic microglial cells following anterograde degeneration of perforant path axons. *Hippocampus* **7,** 341–349 (1997).

58. Bechmann, I., Peter, S., Beyer, M., Gimsa, U. & Nitsch, R. Presence of B-7.2 (CD86) and lack of B7-1 (CD80) on myelin-phagocytosing MHC-II positive rat microglia are associated with nondestructive immunity in vivo. *FASEB J.* 00–0563fje (2001). doi:10.1096/fj.00-0563fje

59. Schluesener, H. J. & Wekerle, H. In vitro selection of permanent T lymphocyte lines with receptors for myelin basic protein (MBP). *Prog. Clin. Biol. Res* **146,** 285–290 (1984).

60. Donnelly, D. J. & Popovich, P. G. Inflammation and its role in neuroprotection, axonal regeneration and functional recovery after spinal cord injury. *Experimental Neurology* **209,** 378–388 (2008).

61. Brown, G. & Neher, J. Inflammatory Neurodegeneration and Mechanisms of Microglial Killing of Neurons. *Molecular Neurobiology* **41,** 242–247 (2010).

62. Napoli, I. & Neumann, H. Protective effects of microglia in multiple sclerosis. *Experimental Neurology* **225,** 24–28 (2010).

63. Glezer, I., Simard, A. R. & Rivest, S. Neuroprotective role of the innate immune system by microglia. *Neuroscience* **147,** 867–883 (2007).

64. Ponomarev, E. D., Maresz, K., Tan, Y. & Dittel, B. N. CNS-Derived Interleukin-4 Is Essential for the Regulation of Autoimmune Inflammation and Induces a State of Alternative Activation in Microglial Cells. *J. Neurosci.* **27,** 10714–10721 (2007).

65. Kapsenberg, M. L. Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol* **3,** 984–993 (2003).

66. Study on cellular events in postthymectomy autoimmune oophoritis in mice. I. Requirement of Lyt-1 effector cells for oocytes damage after adoptive transfer. *J Exp Med* **156,** 1565–1576 (1982).

67. Sakaguchi, S. & Powrie, F. Emerging Challenges in Regulatory T Cell Function and Biology. *Science* **317,** 627–629 (2007).

68. Zhu, J. & Paul, W. E. CD4 T cells: fates, functions, and faults. *Blood* **112,** 1557–1569 (2008).

69. Korn, T., Bettelli, E., Oukka, M. & Kuchroo, V. K. IL-17 and Th17 Cells. *Annual Review of Immunology* **27,** 485–517 (2009).

70. Hickey, W. F. Leukocyte traffic in the central nervous system: the participants and their roles. *Seminars in Immunology* **11,** 125–137 (1999).

71. Aloisi, F., Ria, F. & Adorini, L. Regulation of T-cell responses by CNS antigen-presenting cells: different roles for microglia and astrocytes. *Immunology Today* **21,** 141–147 (2000).

72. Olson, J. K., Girvin, A. M. & Miller, S. D. Direct Activation of Innate and Antigen-Presenting Functions of Microglia following Infection with Theiler's Virus. *J. Virol.* **75,** 9780–9789 (2001).

73. Aloisi, F. *et al.* Relative efficiency of microglia, astrocytes, dendritic cells and B cells in naive CD4+ T cell priming and Th1/Th2 cell restimulation. *European Journal of Immunology* **29,** 2705–2714 (1999).

74. Aloisi, F., Simone, R. D., Columba-Cabezas, S. & Levi, G. Opposite effects of interferon-? and prostaglandin E2 on tumor necrosis factor and interleukin-10

production in microglia: A regulatory loop controlling microglia pro- and anti-inflammatory activities. *J. Neurosci. Res.* **56,** 571–580 (1999).

75. Juedes, A. E. & Ruddle, N. H. Resident and Infiltrating Central Nervous System APCs Regulate the Emergence and Resolution of Experimental Autoimmune Encephalomyelitis. *J Immunol* **166,** 5168–5175 (2001).
76. Gershon, R. K. & Kondo, K. Infectious immunological tolerance. *Immunology* **21,** 903–914 (1971).
77. Miyara, M. & Sakaguchi, S. Natural regulatory T cells: mechanisms of suppression. *Trends in Molecular Medicine* **13,** 108–116 (2007).
78. Barrat, F. J. *et al.* In Vitro Generation of Interleukin 10–producing Regulatory CD4+ T Cells Is Induced by Immunosuppressive Drugs and Inhibited by T Helper Type 1 (Th1)– and Th2-inducing Cytokines. *J Exp Med* **195,** 603–616 (2002).
79. Weiner, H. L. Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells. *Immunological Reviews* **182,** 207–214 (2001).
80. Takahashi, T. *et al.* Immunologic Self-Tolerance Maintained by Cd25+Cd4+Regulatory T Cells Constitutively Expressing Cytotoxic T Lymphocyte–Associated Antigen 4. *J Exp Med* **192,** 303–310 (2000).
81. Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y. & Sakaguchi, S. Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance. *Nature Immunology* **3,** 135–142 (2002).
82. Vigouroux, S., Yvon, E., Biagi, E. & Brenner, M. K. Antigen-induced regulatory T cells. *Blood* **104,** 26–33 (2004).
83. Fontenot, J. D., Gavin, M. A. & Rudensky, A. Y. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nature Immunology* **4,** 330–336 (2003).
84. Hori, S., Nomura, T. & Sakaguchi, S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. *Science* **299,** 1057–1061 (2003).
85. Brunkow, M. E. *et al.* Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nature Genetics* **27,** 68–73 (2001).
86. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T Cells and Immune Tolerance. *Cell* **133,** 775–787 (2008).
87. Mazzolari, E. *et al.* A new case of IPEX receiving bone marrow transplantation. *Bone Marrow Transplantation* **35,** 1033–1034 (2005).
88. Wildin, R., Smyk-Pearson, S. & Filipovich, A. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J Med Genet* **39,** 537–545 (2002).
89. Hori, S., Nomura, T. & Sakaguchi, S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299,** 1057–1061 (2003).
90. Wang, Y. *et al.* Th2 Lymphoproliferative Disorder of LatY136F Mutant Mice Unfolds Independently of TCR-MHC Engagement and Is Insensitive to the Action of Foxp3+ Regulatory T Cells. *J Immunol* **180,** 1565–1575 (2008).
91. Bettelli, E. *et al.* Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* **441,** 235–238 (2006).
92. Lee, Y. K., Mukasa, R., Hatton, R. D. & Weaver, C. T. Developmental plasticity of Th17 and Treg cells. *Current Opinion in Immunology* **21,** 274–280 (2009).

93. Zhou, L. *et al.* TGF-β-induced Foxp3 inhibits TH17 cell differentiation by antagonizing RORγt function. *Nature* **453,** 236–240 (2008).
94. Allan, S. E. *et al.* Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *Int. Immunol.* **19,** 345–354 (2007).
95. Wang, J., Ioan-Facsinay, A., van der Voort, E. I. H., Huizinga, T. W. J. & Toes, R. E. M. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *European Journal of Immunology* **37,** 129–138 (2007).
96. Shevach, E. M. CD4+CD25+ suppressor T cells: more questions than answers. *Nature Reviews Immunology* **2,** 389–400 (2002).
97. Annacker, O. *et al.* CD25+ CD4+ T Cells Regulate the Expansion of Peripheral CD4 T Cells Through the Production of IL-10. *J Immunol* **166,** 3008–3018 (2001).
98. Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. & Powrie, F. An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation. *J Exp Med* **190,** 995–1004 (1999).
99. Ostroukhova, M. *et al.* Tolerance induced by inhaled antigen involves CD4+ T cells expressing membrane-bound TGF-β and FOXP3. *J Clin Invest* **114,** 28–38 (2004).
100. Suciu-Foca, N., Manavalan, J. S. & Cortesini, R. Generation and function of antigen-specific suppressor and regulatory T cells. *Transplant Immunology* **11,** 235–244 (2003).
101. Von Boehmer, H. Mechanisms of suppression by suppressor T cells. *Nat Immunol* **6,** 338–344 (2005).
102. Weiner, H. L. Oral tolerance: immune mechanisms and the generation of Th3-type TGF-beta-secreting regulatory cells. *Microbes and Infection* **3,** 947–954 (2001).
103. Faria, A. M. C. & Weiner, H. L. Oral tolerance. *Immunological Reviews* **206,** 232–259 (2005).
104. Chen, Y., Kuchroo, V. K., Inobe, J., Hafler, D. A. & Weiner, H. L. Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* **265,** 1237–1240 (1994).
105. Kretschmer, K. *et al.* Inducing and expanding regulatory T cell populations by foreign antigen. *Nature Immunology* **6,** 1219–1227 (2005).
106. Weber, M. S. *et al.* Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. *Nat Med* **13,** 935–943 (2007).
107. Jiang, S., Camara, N., Lombardi, G. & Lechler, R. I. Induction of allopeptide-specific human CD4+CD25+ regulatory T cells ex vivo. *Blood* **102,** 2180–2186 (2003).
108. Earle, K. E. *et al.* In vitro expanded human CD4+CD25+ regulatory T cells suppress effector T cell proliferation. *Clinical Immunology* **115,** 3–9 (2005).
109. Hoffmann, P., Eder, R., Kunz-Schughart, L. A., Andreesen, R. & Edinger, M. Large-scale in vitro expansion of polyclonal human CD4+CD25high regulatory T cells. *Blood* **104,** 895–903 (2004).
110. McGeachy, M. J., Stephens, L. A. & Anderton, S. M. Natural Recovery and Protection from Autoimmune Encephalomyelitis: Contribution of CD4+CD25+ Regulatory Cells Within the Central Nervous System. *J Immunol* **175,** 3025–3032 (2005).
111. Korn, T. *et al.* Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med* **13,** 423–431 (2007).
112. Liesz, A. *et al.* Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. *Nat Med* **15,** 192–199 (2009).

113. Bettelli, E. *et al.* IL-10 Is Critical in the Regulation of Autoimmune Encephalomyelitis as Demonstrated by Studies of IL-10- and IL-4-Deficient and Transgenic Mice. *J Immunol* **161,** 3299–3306 (1998).
114. Kennedy, M. K., Torrance, D. S., Picha, K. S. & Mohler, K. M. Analysis of Cytokine mRNA Expression in the Central Nervous System of Mice with Experimental Autoimmune Encephalomyelitis Reveals That IL-10 mRNA Expression Correlates with Recovery. *J Immunol* **149,** 2496–2505 (1992).
115. Kohm, A. P., Carpentier, P. A., Anger, H. A. & Miller, S. D. Cutting Edge: CD4+CD25+ Regulatory T Cells Suppress Antigen-Specific Autoreactive Immune Responses and Central Nervous System Inflammation During Active Experimental Autoimmune Encephalomyelitis. *J Immunol* **169,** 4712–4716 (2002).
116. Zhang, X. *et al.* IL-10 Is Involved in the Suppression of Experimental Autoimmune Encephalomyelitis by CD25+CD4+ Regulatory T Cells. *Int. Immunol.* **16,** 249–256 (2004).
117. De Camargo Furtado, G. *et al.* Regulatory T cells in spontaneous autoimmune encephalomyelitis. *Immunological Reviews* **182,** 122–134 (2001).
118. Wolf, S. A., Gimsa, U., Bechmann, I. & Nitsch, R. Differential expression of costimulatory molecules B7-1 and B7-2 on microglial cells induced by Th1 and Th2 cells in organotypic brain tissue. *Glia* **36,** 414–420 (2001).
119. Mutlu, L. *et al.* Tolerogenic effect of fiber tract injury: reduced EAE severity following entorhinal cortex lesion. *Exp Brain Res* **178,** 542–553 (2007).
120. Bechmann, I. & Nitsch, R. Astrocytes and microglial cells incorporate degenerating fibers following entorhinal lesion: A light, confocal, and electron microscopical study using a phagocytosis-dependent labeling technique. *Glia* **20,** 145–154 (1997).
121. Schulze-Topphoff, U. *et al.* Activation of kinin receptor B1 limits encephalitogenic T lymphocyte recruitment to the central nervous system. *Nat Med* **15,** 788–793 (2009).
122. Fitch, F. W., Gajewski, T. F. & Hu-Li, J. in *Current Protocols in Immunology* 3.13.1–3.13.15 (John Wiley & Sons, Inc., 2006). at <http://onlinelibrary.wiley.com/doi/10.1002/0471142735.im0313s72/abstract>
123. Nolte, C., Moller, T., Walter, T. & Kettenmann, H. Complement 5a controls motility of murine microglial cells in vitro via activation of an inhibitory G-protein and the rearrangement of the actin cytoskeleton. *Neuroscience* **73,** 1091–1107 (1996).
124. Baron, U. *et al.* DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *European Journal of Immunology* **37,** 2378–2389 (2007).
125. Frei, K. *et al.* Antigen presentation and tumor cytotoxicity by interferon-γ-treated microglial cells. *European Journal of Immunology* **17,** 1271–1278 (1987).
126. Floess, S. *et al.* Epigenetic Control of the foxp3 Locus in Regulatory T Cells. *PLoS Biol* **5,** e38 (2007).
127. Hofman, F. M. *et al.* Immunoregulatory Molecules and IL 2 Receptors Identified in Multiple Sclerosis Brain. *J Immunol* **136,** 3239–3245 (1986).
128. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J Exp Med* **182,** 1985–1996 (1995).
129. Nagahama, K. *et al.* Differential Control of Allo-Antigen-Specific Regulatory T Cells and Effector T Cells by Anti-CD4 and Other Agents in Establishing Transplantation Tolerance. *Int. Immunol.* **21,** 379–391 (2009).

130. Koppelman, B., Neefjes, J., Devries, J. & Dewaalmalefyt, R. Interleukin-10 Down-Regulates MHC Class II αβ Peptide Complexes at the Plasma Membrane of Monocytes by Affecting Arrival and Recycling. *Immunity* **7,** 861–871 (1997).
131. Dalpke, A. H. *et al.* Immunostimulatory CpG-DNA Activates Murine Microglia. *J Immunol* **168,** 4854–4863 (2002).
132. Santambrogio, L. *et al.* Developmental plasticity of CNS microglia. *PNAS* **98,** 6295–6300 (2001).
133. Xu, J. & Ling, E. A. Upregulation and induction of surface antigens with special reference to MHC class II expression in microglia in postnatal rat brain following intravenous or intraperitoneal injections of lipopolysaccharide. *J Anat* **184,** 285–296 (1994).
134. Marguerat, S., MacDonald, H. R., Kraehenbuhl, J.-P. & van Meerwijk, J. P. M. Protection from Radiation-Induced Colitis Requires MHC Class II Antigen Expression by Cells of Hemopoietic Origin. *J Immunol* **163,** 4033–4040 (1999).
135. Ousman, S. S. & Kubes, P. Immune surveillance in the central nervous system. *Nature Neuroscience* **15,** 1096–1101 (2012).
136. Rossi, B., Angiari, S., Zenaro, E., Budui, S. L. & Constantin, G. Vascular inflammation in central nervous system diseases: adhesion receptors controlling leukocyte–endothelial interactions. *J Leukoc Biol* **89,** 539–556 (2011).
137. Schwartz, M. & Shechter, R. Protective autoimmunity functions by intracranial immunosurveillance to support the mind: The missing link between health and disease. *Molecular Psychiatry* **15,** 342–354 (2010).
138. Vajkoczy, P., Laschinger, M. & Engelhardt, B. α4-integrin-VCAM-1 binding mediates G protein–independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. *J Clin Invest* **108,** 557–565 (2001).
139. Xu, H. *et al.* Requirements for passage of T lymphocytes across non-inflamed retinal microvessels. *Journal of Neuroimmunology* **142,** 47–57 (2003).
140. Bartholomaus, I. *et al.* Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions. *Nature* **462,** 94–98 (2009).
141. Brabb, T. *et al.* In Situ Tolerance within the Central Nervous System as a Mechanism for Preventing Autoimmunity. *J Exp Med* **192,** 871–880 (2000).
142. Stamatovic, S. M., Keep, R. F. & Andjelkovic, A. V. Brain Endothelial Cell-Cell Junctions: How to 'Open' the Blood Brain Barrier. *Curr Neuropharmacol* **6,** 179–192 (2008).
143. Wilson, E. H., Weninger, W. & Hunter, C. A. Trafficking of immune cells in the central nervous system. *Journal of Clinical Investigation* **120,** 1368–1379 (2010).
144. Kaminski, M. *et al.* Migration of monocytes after intracerebral injection at entorhinal cortex lesion site. *J. Leukoc. Biol.* **92,** 31–39 (2012).
145. Bhat, R. & Steinman, L. Innate and adaptive autoimmunity directed to the central nervous system. *Neuron* **64,** 123–132 (2009).
146. Hanisch, U.-K. Microglia as a source and target of cytokines. *Glia* **40,** 140–155 (2002).
147. Felger, J. C. *et al.* Brain dendritic cells in ischemic stroke: Time course, activation state, and origin. *Brain, Behavior, and Immunity* **In Press, Uncorrected Proof,**
148. Li, H.-L. *et al.* IL-17 and IFN-γ mRNA expression is increased in the brain and systemically after permanent middle cerebral artery occlusion in the rat. *Journal of Neuroimmunology* **116,** 5–14 (2001).

149. Tanga, F. Y., Nutile-McMenemy, N. & DeLeo, J. A. The CNS role of Toll-like receptor 4 in innate neuroimmunity and painful neuropathy. *Proc Natl Acad Sci U S A* **102,** 5856–5861 (2005).

150. Neumann, E., Lefèvre, S., Zimmermann, B., Gay, S. & Müller-Ladner, U. Rheumatoid arthritis progression mediated by activated synovial fibroblasts. *Trends in Molecular Medicine* **In Press, Corrected Proof,**

151. Mäkelä, J., Koivuniemi, R., Korhonen, L. & Lindholm, D. Interferon-γ Produced by Microglia and the Neuropeptide PACAP Have Opposite Effects on the Viability of Neural Progenitor Cells. *PLoS One* **5,** (2010).

152. Boehm, U., Klamp, T., Groot, M. & Howard, J. C. CELLULAR RESPONSES TO INTERFERON-γ. *Annual Review of Immunology* **15,** 749–795 (1997).

153. Platanias, L. C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* **5,** 375–386 (2005).

154. Gottfried-Blackmore, A. *et al.* Acute in vivo exposure to interferon-γ enables resident brain dendritic cells to become effective antigen presenting cells. *Proceedings of the National Academy of Sciences* **106,** 20918–20923 (2009).

155. Selvaraj, R. K. & Geiger, T. L. A Kinetic and Dynamic Analysis of Foxp3 Induced in T Cells by TGF-beta. *J Immunol* **178,** 7667–7677 (2007).

156. Simpson, J. ., Newcombe, J., Cuzner, M. . & Woodroofe, M. . Expression of monocyte chemoattractant protein-1 and other β-chemokines by resident glia and inflammatory cells in multiple sclerosis lesions. *Journal of Neuroimmunology* **84,** 238–249 (1998).

157. Juedes, A. E., Hjelmström, P., Bergman, C. M., Neild, A. L. & Ruddle, N. H. Kinetics and Cellular Origin of Cytokines in the Central Nervous System: Insight into Mechanisms of Myelin Oligodendrocyte Glycoprotein-Induced Experimental Autoimmune Encephalomyelitis. *J Immunol* **164,** 419–426 (2000).

158. Berman, J. W., Guida, M. P., Warren, J., Amat, J. & Brosnan, C. F. Localization of monocyte chemoattractant peptide-1 expression in the central nervous system in experimental autoimmune encephalomyelitis and trauma in the rat. *J. Immunol.* **156,** 3017–3023 (1996).

159. D'Aversa, T. G., Weidenheim, K. M. & Berman, J. W. CD40-CD40L Interactions Induce Chemokine Expression by Human Microglia. *Am J Pathol* **160,** 559–567 (2002).

160. Ransohoff, R. M. & Tani, M. Do chemokines mediate leukocyte recruitment in post-traumatic CNS inf lammation? *Trends in Neurosciences* **21,** 154–159 (1998).

161. Asensio, V. C. & Campbell, I. L. Chemokines in the CNS: plurifunctional mediators in diverse states. *Trends in Neurosciences* **22,** 504–512 (1999).

162. Karpus, W. J. & Kennedy, K. J. MIP-1alpha and MCP-1 differentially regulate acute and relapsing autoimmune encephalomyelitis as well as Th1/Th2 lymphocyte differentiation. *J Leukoc Biol* **62,** 681–687 (1997).

163. Luther, S. A. & Cyster, J. G. Chemokines as regulators of T cell differentiation. *Nature Immunology* **2,** 102–107 (2001).

164. Yang, M.-S. *et al.* Interleukin-13 and -4 induce death of activated microglia. *Glia* **38,** 273–280 (2002).

165. Aloisi, F., Ria, F. & Adorini, L. Regulation of T-cell responses by CNS antigen-presenting cells: different roles for microglia and astrocytes. *Immunology Today* **21,** 141–147 (2000).

166. Louten, J., Boniface, K. & de Waal Malefyt, R. Development and function of TH17 cells in health and disease. *Journal of Allergy and Clinical Immunology* **123,** 1004–1011 (2009).
167. McGeachy, M. J. *et al.* Interleukin 23 receptor is essential for terminal differentiation of effector T helper type 17 cells in vivo. *Nat Immunol* **10,** 314–324 (2009).
168. McGeachy, M. J. *et al.* TGF-[beta] and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain TH-17 cell-mediated pathology. *Nat Immunol* **8,** 1390–1397 (2007).
169. Petermann, F. & Korn, T. Cytokines and effector T cell subsets causing autoimmune CNS disease. *FEBS Letters* **585,** 3747–3757 (2011).
170. Jäger, A., Dardalhon, V., Sobel, R. A., Bettelli, E. & Kuchroo, V. K. Th1, Th17, and Th9 Effector Cells Induce Experimental Autoimmune Encephalomyelitis with Different Pathological Phenotypes. *J Immunol* **183,** 7169–7177 (2009).
171. Domingues, H. S., Mues, M., Lassmann, H., Wekerle, H. & Krishnamoorthy, G. Functional and pathogenic differences of Th1 and Th17 cells in experimental autoimmune encephalomyelitis. *PLoS ONE* **5,** e15531 (2010).
172. Murphy, K. M. & Stockinger, B. Effector T cell plasticity: flexibility in the face of changing circumstances. *Nat Immunol* **11,** 674–680 (2010).
173. Zhou, L., Chong, M. M. W. & Littman, D. R. Plasticity of CD4+ T Cell Lineage Differentiation. *Immunity* **30,** 646–655 (2009).
174. Lee, Y. K. *et al.* Late Developmental Plasticity in the T Helper 17 Lineage. *Immunity* **30,** 92–107 (2009).
175. Hegazy, A. N. *et al.* Interferons Direct Th2 Cell Reprogramming to Generate a Stable GATA-3+T-bet+ Cell Subset with Combined Th2 and Th1 Cell Functions. *Immunity* **32,** 116–128 (2010).
176. Hirota, K. *et al.* Fate mapping of interleukin 17-producing T cells in inflammatory responses. *Nat Immunol* **12,** 255–263 (2011).
177. Kurschus, F. C. *et al.* Genetic proof for the transient nature of the Th17 phenotype. *European Journal of Immunology* **40,** 3336–3346 (2010).
178. Collison, L. W. *et al.* Interleukin-35-mediated induction of a novel regulatory T cell population. *Nat Immunol* **11,** 1093–1101 (2010).
179. Roncarolo, M. G. *et al.* Interleukin‐10‐secreting type 1 regulatory T cells in rodents and humans. *Immunological Reviews* **212,** 28–50 (2006).
180. Takahashi, T. *et al.* Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* **10,** 1969–1980 (1998).
181. McHugh, R. S. *et al.* CD4+CD25+ Immunoregulatory T Cells: Gene Expression Analysis Reveals a Functional Role for the Glucocorticoid-Induced TNF Receptor. *Immunity* **16,** 311–323 (2002).
182. Thornton, A. M. & Shevach, E. M. Suppressor Effector Function of CD4+CD25+ Immunoregulatory T Cells Is Antigen Nonspecific. *J Immunol* **164,** 183–190 (2000).
183. Sawada, M., Suzumura, A. & Marunouchi, T. Induction of Functional Interleukin-2 Receptor in Mouse Microglia. *Journal of Neurochemistry* **64,** 1973–1979 (1995).
184. Yamazaki, S. *et al.* Direct Expansion of Functional CD25+ CD4+ Regulatory T Cells by Antigen-processing Dendritic Cells. *J Exp Med* **198,** 235–247 (2003).

185. Xu, D. *et al.* CD4+CD25+ Regulatory T Cells Suppress Differentiation and Functions of Th1 and Th2 Cells, Leishmania major Infection, and Colitis in Mice. *J Immunol* **170,** 394–399 (2003).
186. Reynolds, A. D., Banerjee, R., Liu, J., Gendelman, H. E. & Mosley, R. L. Neuroprotective activities of CD4+CD25+ regulatory T cells in an animal model of Parkinson's disease. *J Leukoc Biol* **82,** 1083–1094 (2007).
187. Ruby, C. E. *et al.* Cutting Edge: OX40 Agonists Can Drive Regulatory T Cell Expansion if the Cytokine Milieu Is Right. *The Journal of Immunology* **183,** 4853 –4857 (2009).
188. Ansel, K. M., Lee, D. U. & Rao, A. An epigenetic view of helper T cell differentiation. *Nature Immunology* **4,** 616–623 (2003).
189. Gordon, S. Alternative activation of macrophages. *Nature Reviews Immunology* **3,** 23–35 (2003).
190. Riquelme, P. *et al.* IFN-γ-induced iNOS Expression in Mouse Regulatory Macrophages Prolongs Allograft Survival in Fully Immunocompetent Recipients. *Molecular Therapy* doi:10.1038/mt.2012.168
191. Hutchinson, J. A. *et al.* Cutting Edge: Immunological Consequences and Trafficking of Human Regulatory Macrophages Administered to Renal Transplant Recipients. *J Immunol* **187,** 2072–2078 (2011).
192. Yong, V. W. & Rivest, S. Taking Advantage of the Systemic Immune System to Cure Brain Diseases. *Neuron* **64,** 55–60 (2009).
193. Chan, W. K. *et al.* MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-γ challenge. *Experimental Hematology* **36,** 1545–1555 (2008).
194. Ljunggren, G. & Anderson, D. J. Cytokine induced modulation of MHC class I and class II molecules on human cervical epithelial cells. *Journal of Reproductive Immunology* **38,** 123–138 (1998).
195. Guillemin, G. J. & Brew, B. J. Microglia, macrophages, perivascular macrophages, and pericytes: a review of function and identification. *J Leukoc Biol* **75,** 388–397 (2004).
196. Carson, M. J., Reilly, C. R., Sutcliffe, J. G. & Lo, D. Mature microglia resemble immature antigen-presenting cells. *Glia* **22,** 72–85 (1998).
197. Sheffield, L. G. & Berman, N. E. J. Microglial Expression of MHC Class II Increases in Normal Aging of Nonhuman Primates. *Neurobiology of Aging* **19,** 47–55 (1998).
198. VanGuilder, H. D. *et al.* Concurrent hippocampal induction of MHC II pathway components and glial activation with advanced aging is not correlated with cognitive impairment. *J Neuroinflammation* **8,** 138 (2011).
199. Streit, W. J. Microglial Response to Brain Injury: A Brief Synopsis. *Toxicologic Pathology* **28,** 28 –30 (2000).
200. Schmitt, A. B. *et al.* Major histocompatibility complex class II expression by activated microglia caudal to lesions of descending tracts in the human spinal cord is not associated with a T cell response. *Acta Neuropathol* **100,** 528–536 (2000).
201. Stubbe, T. *et al.* Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO. *Journal of Cerebral Blood Flow & Metabolism* **33,** 37–47 (2013).
202. Singh, B. *et al.* Control of intestinal inflammation by regulatory T cells. *Immunological Reviews* **182,** 190–200 (2001).

203. Walker, L. S. K. CD4+ CD25+ Treg: divide and rule? *Immunology* **111,** 129–137 (2004).
204. Gavin, M. A., Clarke, S. R., Negrou, E., Gallegos, A. & Rudensky, A. Homeostasis and anergy of CD4+CD25+ suppressor T cells in vivo. *Nature Immunology* **3,** 33–41 (2001).
205. Bender, J., Mitchell, T., Kappler, J. & Marrack, P. Cd4+ T Cell Division in Irradiated Mice Requires Peptides Distinct from Those Responsible for Thymic Selection. *J Exp Med* **190,** 367–374 (1999).
206. Almeida, A. R. M., Legrand, N., Papiernik, M. & Freitas, A. A. Homeostasis of Peripheral CD4+ T Cells: IL-2Rα and IL-2 Shape a Population of Regulatory Cells That Controls CD4+ T Cell Numbers. *J Immunol* **169,** 4850–4860 (2002).
207. Soulet, D. & Rivest, S. Bone-marrow-derived microglia: myth or reality? *Current Opinion in Pharmacology* **8,** 508–518 (2008).
208. Trnovec, T., Kállay, Z. & Bezek, Š. Effects of ionizing radiation on the blood brain barrier permeability to pharmacologically active substances. *International Journal of Radiation Oncology*Biology*Physics* **19,** 1581–1587 (1990).
209. Ransohoff, R. M. & Estes, M. L. Astrocyte expression of major histocompatibility complex gene products in multiple sclerosis brain tissue obtained by stereotactic biopsy. *Arch. Neurol.* **48,** 1244–1246 (1991).
210. Balabanov, R., Beaumont, T. & Dore-Duffy, P. Role of central nervous system microvascular pericytes in activation of antigen-primed splenic T-lymphocytes. *Journal of Neuroscience Research* **55,** 578–587 (1999).
211. Mantovani, A., Sozzani, S., Locati, M., Allavena, P. & Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in Immunology* **23,** 549–555 (2002).
212. Tan, J. *et al.* Activation of microglial cells by the CD40 pathway: relevance to multiple sclerosis. *Journal of Neuroimmunology* **97,** 77–85 (1999).
213. Becher, B., Durell, B. G., Miga, A. V., Hickey, W. F. & Noelle, R. J. The Clinical Course of Experimental Autoimmune Encephalomyelitis and Inflammation Is Controlled by the Expression of Cd40 within the Central Nervous System. *J. Exp. Med.* **193,** 967–974 (2001).
214. Becher, B., Blain, M. & Antel, J. P. CD40 engagement stimulates IL-12 p70 production by human microglial cells: basis for Th1 polarization in the CNS. *Journal of Neuroimmunology* **102,** 44–50 (2000).
215. Gelderblom, M. *et al.* Temporal and Spatial Dynamics of Cerebral Immune Cell Accumulation in Stroke. *Stroke* **40,** 1849–1857 (2009).
216. Windhagen, A. *et al.* Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J Exp Med* **182,** 1985–1996 (1995).
217. Sellebjerg, F., Jensen, J. & Ryder, L. P. Costimulatory CD80 (B7-1) and CD86 (B7-2) on cerebrospinal fluid cells in multiple sclerosis. *Journal of Neuroimmunology* **84,** 179–187 (1998).
218. Khan, N., Gowthaman, U., Pahari, S. & Agrewala, J. N. Manipulation of Costimulatory Molecules by Intracellular Pathogens: Veni, Vidi, Vici!! *PLoS Pathog* **8,** e1002676 (2012).
219. Keir, M. E., Butte, M. J., Freeman, G. J. & Sharpe, A. H. PD-1 and Its Ligands in Tolerance and Immunity. *Annu. Rev. Immunol.* **26,** 677–704 (2008).

220. Magnus, T. *et al.* Microglial Expression of the B7 Family Member B7 Homolog 1 Confers Strong Immune Inhibition: Implications for Immune Responses and Autoimmunity in the CNS. *J. Neurosci.* **25,** 2537–2546 (2005).
221. Chastain, E. M. L., Duncan, D. S., Rodgers, J. M. & Miller, S. D. The Role of Antigen Presenting Cells in Multiple Sclerosis. *Biochim Biophys Acta* **1812,** 265–274 (2011).
222. Tierney, J. B., Kharkrang, M. & La Flamme, A. C. Type II-activated macrophages suppress the development of experimental autoimmune encephalomyelitis. *Immunol Cell Biol* **87,** 235–240 (2008).
223. Häusler, K. G. *et al.* Interferon-γ differentially modulates the release of cytokines and chemokines in lipopolysaccharide- and pneumococcal cell wall-stimulated mouse microglia and macrophages. *European Journal of Neuroscience* **16,** 2113–2122 (2002).
224. Mahnke, K., Bedke, T. & Enk, A. H. Regulatory conversation between antigen presenting cells and regulatory T cells enhance immune suppression. *Cellular Immunology* **250,** 1–13 (2007).
225. Cunha, F. Q., Moncada, S. & Liew, F. Y. Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon-gamma in murine macrophages. *Biochem. Biophys. Res. Commun.* **182,** 1155–1159 (1992).
226. Gratchev, A. *et al.* Mφ1 and Mφ2 can be re-polarized by Th2 or Th1 cytokines, respectively, and respond to exogenous danger signals. *Immunobiology* **211,** 473–486 (2006).
227. Mosser, D. M. The many faces of macrophage activation. *J Leukoc Biol* **73,** 209–212 (2003).
228. McQuillan, K., Lynch, M. A. & Mills, K. H. G. Activation of mixed glia by Aβ-specific Th1 and Th17 cells and its regulation by Th2 cells. *Brain, Behavior, and Immunity* **24,** 598–607 (2010).
229. Hu, X. *et al.* Sensitization of IFN-γ Jak-STAT signaling during macrophage activation. *Nature Immunology* **3,** 859–866 (2002).
230. Kawakami, N. *et al.* The Activation Status of Neuroantigen-specific T Cells in the Target Organ Determines the Clinical Outcome of Autoimmune Encephalomyelitis. *J Exp Med* **199,** 185–197 (2004).
231. Wang, Y.-C. *et al.* Notch Signaling Determines the M1 versus M2 Polarization of Macrophages in Antitumor Immune Responses. *Cancer Res* **70,** 4840–4849 (2010).

6 Appendix

List of Figures

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Publications

Ebner F., Stubbe T., Brandt C., Siffrin V., Nitsch R., Sawitzki B.
MHCII-dependent accumulation of regulatory T cells in the lesioned CNS.
(in preparation)

Ebner F., Brandt C., Thiele P., Richter D., Siffrin V., Schüler J., Stubbe T., Ellinghaus A., Meisel C., Sawitzki B., Nitsch R.
Microglial activation milieu controls regulatory T cell responses.
J Immunol. 2013 Dec 1;191(11):5594-602.

Stubbe T., Ebner F., Richter D., Engel O., Klehmet J., Royl G., Meisel A., Nitsch R., Meisel C., Brandt C.
Regulatory T cells accumulate and proliferate in the ischemic hemisphere for up to 30 days after MCAO.
J Cereb Blood Flow Metab. 2013 Jan; 33(1):37-47.

Presentations at meetings and conferences

Ebner F., Thiele P., Sawitzki B., Nitsch R., Brandt C.
Microglia induced regulatory T cells (Tregs) suppress experimental allergic encephalomyelitis (EAE)
Poster presentation at the *7th Forum of European Neuroscience (FENS), 2010*

Ebner F., Thiele P., Sawitzki B., Nitsch R., Brandt C.
Microglia induced regulatory T cells (Tregs) suppress experimental allergic encephalomyelitis (EAE)
Poster presentation (Posterprize) at the *Berlin Neuroscience Forum (BNF), 2010*

Ebner F., Thiele P., Sawitzki B., Nitsch R., Brandt C.
Microglia induced regulatory T cells (Tregs) suppress experimental allergic encephalomyelitis (EAE)
Oral presentation at the *Society for Neuroscience (SfN) Meeting, 2009*

Ebner F., Thiele P., Sawitzki B., Nitsch R., Brandt C.
Microglia induced regulatory T cells (Tregs) suppress EAE
Poster presentation at the *2nd European Congress of Immunology (ECI), 2009*

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Selbständigkeitserklärung

Ich versichere an Eides statt, dass ich die vorliegende Dissertation selbst und ohne unzulässige Hilfe Dritter verfasst habe und die benutzten Hilfsmittel sowie die Literatur vollständig angegeben sind. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde und mir der Inhalt Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt-Universität zu Berlin vom 01.09.2005 bekannt ist.

Berlin,

(Friederike Ebner)